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Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms

(Third Edition)

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1. Page 61, last line: $2(90/n)^2$ should be $2(90/\pi)^2$.
2. Page 205, paragraph 1: The program is written in BASIC PLUS 2, Version 1.61, rather than in FORTRAN.

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March 1985

METHODS FOR MEASURING THE ACUTE TOXICITY OF EFFLUENTS
TO FRESHWATER AND MARINE ORGANISMS

(Third Edition)

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1985

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Environmental measurements are required to determine the quality of ambient water, the character of effluents, and the effects of pollutants on aquatic life. EMSL-Cincinnati conducts research to develop, evaluate, and promulgate methods to:

- Measure the presence and concentration of physical, chemical and radiological pollutants in water, wastewater, bottom sediments, and solid waste.
- Concentrate, recover, and identify enteric viruses, bacteria, and other microorganisms in water.
- Measure the effects of pollution on freshwater, estuarine, and marine organisms, including the phytoplankton, zooplankton, periphyton, macrophyton, macroinvertebrates, and fish.
- Automate the measurement of the physical, chemical, and biological quality of water.
- Conduct an Agencywide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

The Federal Water Pollution Control Act Amendments (Clean Water Act or CWA) of 1977 (PL 95-217) explicitly state that it is the national policy that the discharge of toxic substances in toxic amounts be prohibited. Determination of the toxicity of effluents, therefore, plays an important role in identifying and controlling toxic discharges to surface waters. This report provides standardized methods for measuring the acute toxicity of effluents to aquatic life, for use by the USEPA regional and state programs, and National Pollutant Discharge Elimination System (NPDES) permittees.

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Support Laboratory - Cincinnati

PREFACE

This manual represents the third edition of the general purpose effluent acute toxicity test manual initially published by EMSL-Cincinnati in January, 1978. This edition of the manual was reviewed by the Bioassay Subcommittee of the EMSL-Cincinnati Biological Advisory Committee, USEPA headquarters and regional staff, state and interstate water pollution control programs, environmental protection groups, trade associations, major industries, consulting firms, academic institutions engaged in aquatic toxicology research, and other interested parties in the private sector.

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ABSTRACT

This manual describes methods for measuring the acute toxicity of effluents to freshwater and marine macroinvertebrates and fish. The methods include a preliminary range-finding test, a screening test, and multi-concentration (definitive) static and flow-through toxicity tests. Also included are guidelines on laboratory safety, quality assurance, facilities and equipment, effluent sampling and holding, dilution water, test species selection and handling, data interpretation and utilization, report preparation, organism culturing, and dilutor and mobile bioassay laboratory design.

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SECTION 1

INTRODUCTION

The Federal Water Pollution Control Act Amendments (Clean Water Act) of 1977 (PL 95-217), Section 101(a)(3), in the Declaration of Goals and Policy, states that "it is the national policy that the discharge of toxic pollutants in toxic amounts be prohibited." Current Agency programs for the control of toxic discharges are based in part on effluent limitations for individual chemicals. However, data on the toxicity of substances to aquatic organisms are available for only a limited number of elements and compounds. Effluent limitations, therefore, may not provide adequate protection for aquatic life where the toxicity of the components in the effluent is not known, where there are additive, synergistic, or antagonistic effects between toxic substances in complex effluents, and/or where a complete chemical characterization of the effluent has not been carried out. Since it is not economically feasible to determine the toxicity of each of the thousands of potentially toxic substances in complex effluents or to conduct exhaustive chemical analyses of effluents, the most direct and cost-effective approach to the measurement of the toxicity of effluents is to conduct an effluent toxicity test with aquatic organisms. For this reason, there has been a steady increase in the use of effluent toxicity tests within the Agency and state NPDES programs to identify toxic discharges, and by permittees as a self-monitoring tool (USEPA, 1979c). This manual was prepared to provide standardized methods for effluent toxicity tests to minimize intralaboratory and interlaboratory variability in toxicity tests conducted by USEPA regional and state programs and NPDES permittees.

The first toxicity test methodology developed specifically for effluents was provided in the previous editions of this manual (Peltier, 1978a,b), which were developed by the Bioassay Subcommittee of the Biological Advisory Committee sponsored by EMSL-Cincinnati. The changes in the membership of the Bioassay Subcommittee (see the Preface) and the content of this manual reflect the considerable additional experience in effluent toxicity testing gained by personnel within the USEPA since the last (July 1978) edition of the manual was published.

The acute toxicity tests for effluents described in this report are based on the cumulative experience of numerous USEPA regional and state effluent toxicity monitoring programs, acquired during the past ten years. These tests are used to determine the effluent concentration, expressed as a percent volume, that is lethal to 50% of the organisms within the prescribed period of time (LC50). Where death is not easily detected, such as with the invertebrates, immobilization is considered equivalent to death. In cases where an effect other than death (or immobilization) is used, the results are expressed in terms of the median effective concentration, or EC50.

Acute toxicity tests are used in the NPDES Permit Program to identify effluents containing toxic wastes discharged in toxic amounts. The data are used to predict potential acute and chronic toxicity in the receiving water, based on the LC50 and appropriate dilution, application, and persistence factors (see Section 12). The tests are conducted as a part of compliance sampling inspections, compliance biomonitoring inspections, performance audit inspections, and during special investigations. The samples are transported to a central laboratory for testing, or the tests are conducted on-site by the regulatory agency or the permittee. Acute toxicity tests can also be used in a toxicity reduction evaluation to identify toxic waste streams within plants, which would aid in the development and implementation of toxicity reduction plans, and can be used to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA 1984b).

Two types of acute toxicity tests are utilized -- static and flow-through. The selection of the test type will depend upon the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity. Special environmental requirements of some organisms, such as flowing water, or fluctuating water levels, may preclude the use of static tests.

A negative result from an acute toxicity test with a given effluent sample does not preclude the presence of chronic toxicity. Also, because of the potential temporal variability in the toxicity of wastes, a negative result with a particular sample does not preclude the possibility that samples collected from that discharge at some other time might exhibit acute (or chronic) toxicity.

The frequency with which these toxicity tests are conducted on an effluent under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

STATIC TESTS

Types of static tests include: (1) nonrenewal - the test organisms are exposed to the same effluent solution for the duration of the test; and (2) renewal - the test organisms are exposed to a fresh solution of the same concentration of effluent every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of the effluent solution in the test chambers. Because of toxicant adsorption on the test chambers, uptake by test organisms and the effect of metabolites on toxicity, the renewal system is preferred.

The advantages and disadvantages of static tests are as follows (also see Buikema et al, 1982; Stephan, 1982):

Advantages:

1. Simple and inexpensive
2. Very cost effective.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many sequential tests on samples collected over time.
4. Small volume (1 to 20 L) of effluent required.
5. Provides some measure of persistence of toxicity, i.e. aging of the effluent (nonrenewal static test).

Disadvantages:

1. Results do not reflect temporal changes in effluent toxicity.
2. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
3. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
4. Generally less sensitive than flow-through test, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

FLOW-THROUGH TESTS

Two types of flow-through tests are in common use: (1) effluent is pumped continuously from the sampling point directly to the dilutor system; and (2) effluent grab or composite samples are collected periodically, placed in a tank adjacent to the test laboratory, and pumped continuously from the tank to the dilutor system. The flow-through method employing continuous effluent sampling is the preferred method for on-site tests. Because of the large volume (often 400 L/day) of effluent normally required for flow-through tests, it is generally considered too costly and impractical to conduct these tests off-site at a central laboratory. However, a mini-dilutor requires substantially smaller volumes and may be practical to use off-site (Benoit et al., 1982).

The advantages and disadvantages of flow-through tests are as follows:

Advantages:

1. Provide a more representative evaluation of effluent acute toxicity, especially if toxicity varies with time.
2. DO concentrations are more easily maintained in the test chambers.
3. A higher loading factor (biomass) may be used.
4. The possibility of loss of toxicant due to volatilization, adsorption, degradation and uptake is reduced.

Disadvantages:

1. Large volumes of effluent and dilution water are required.
2. Test equipment is more complex and expensive, and requires more maintenance and attention.
3. More space is required to conduct tests.
4. Does not measure the effect of aging of the waste on toxicity.
5. Because of resources required, it would be very difficult to perform multiple or overlapping sequential tests.

TEST FOR PERSISTENCE OF EFFLUENT TOXICITY

The persistence of the toxicity of an effluent is of interest in assessing the potential effects of the effluent on the receiving water. If the toxicity of the waste has a half-life of less than 96 h, the toxicity can be considered "non-persistent" (p. 122, USEPA, 1973). Methods for determining the persistence of toxicity are discussed on p. 38.

SECTION 2

HEALTH AND SAFETY¹

GENERAL PRECAUTIONS

Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should protect themselves from injury by taking all safety precautions necessary for the prevention of bodily injury, or inhalation or absorption of corrosive or toxic substances through skin contact, and the prevention of asphyxiation due to lack of oxygen or presence of noxious gases.

Prior to sample collection and laboratory work, personnel should make sure that all necessary safety equipment and materials have been obtained and are in good condition.

SAFETY EQUIPMENT

1. Personal Safety Gear

Personnel should use safety equipment, as required, such as rubber aprons, lab coats, respirators, gloves, safety glasses, hard hat, and safety shoes.

2. Laboratory Safety Equipment

Each laboratory (including mobile laboratories) should have safety equipment such as first aid kits, fire extinguishers, fire blankets, etc.

Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

GENERAL LABORATORY AND FIELD OPERATIONS

1. Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials. It is recommended that personnel collecting samples and carrying out toxicity tests should not work alone.
2. Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized.

¹Adapted from: USEPA, 1979c.

3. It is advisable to cleanse exposed parts of the body with soap and water immediately after collecting effluent samples.
4. All containers should be adequately labeled to indicate their contents.
5. Good housekeeping contributes to safety and reliable results.
6. Electrical equipment or cords not having Underwriters Laboratories approval should not be used. Ground-fault interrupters should be installed in all "wet" labs where electrical equipment is used.
7. Mobile laboratories should be properly grounded to protect against electrical shock.
8. Staff training in basic first aid and cardio-pulmonary resuscitation is strongly recommended.

DISEASE PREVENTION

Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, and polio.

SAFETY MANUALS

For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, see permittee and general industrial safety manuals, including USEPA, 1977, and Walters and Jameson, 1984.

SECTION 3

QUALITY ASSURANCE¹

Quality assurance (QA) practices for effluent toxicity tests include all aspects of the test that affect the accuracy and precision of the data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) use of reference toxicants; (7) record keeping; and (8) data evaluation. For general guidance on good laboratory practices related to toxicity testing, see: FDA, 1978; USEPA, 1979d, 1980a, and 1980b; and DeWoskin, 1984.

EFFLUENT SAMPLING AND HANDLING

If effluent samples are collected for off-site testing, the samples should be preserved as described in Section 7.

TEST ORGANISMS

Test organisms listed in Table 1 (Section 5) may be used. The organisms should be free of disease, as indicated by minimal rates of mortality in holding tanks and in test controls, should not have been exposed to pollutants or other stress prior to testing, and are to be in an early, life stage, as specified in Table 1.

FACILITIES, EQUIPMENT, AND TEST CHAMBERS

Laboratory and bioassay temperature control equipment must be adequate to maintain recommended test water temperatures. Thermometers, pH meters, dilutors, and other measuring devices must be calibrated by following the manufacturer's recommended methods before, and at appropriate intervals during, use. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 4).

DILUTION WATER

The dilution water used in the tests will depend on the objectives of the study and logistical constraints. Receiving water collected upstream from the discharge point should be used as dilution water wherever possible. Depending upon the objectives, it may be desirable to use receiving water as the dilution water even when it is contaminated by "upstream" sources. These objectives may include determination of the toxicity of the receiving water upstream from the discharge and the additive toxicity of the waste and the contaminated intake water used for plant processes. If the presence of contaminants in the receiving water makes it unsuitable as dilution water, other uncontaminated surface water such as a nearby tributary, or ground water, or synthetic water may be employed.

¹Adapted from: USEPA, 1979c.

TEST CONDITIONS

Water temperature ranges for the test organisms (Table 1) must be maintained within the recommended limits. Dissolved oxygen concentrations should be checked at the beginning of the test and regularly throughout the test period. Aeration is employed if necessary to ensure that the DO does not fall below 40% saturation for warm-water species and 60% saturation for cold-water species.

REFERENCE TOXICANTS

A reference toxicant is to be used to establish the validity of effluent toxicity data generated by toxicity test laboratories. Three reference toxicants, sodium dodecylsulfate, sodium pentachlorophenate, and cadmium chloride, are available from EMSL-Cincinnati. Instructions for their use and the expected LC50 values are provided with the samples.

Factors affecting the accuracy of the data include test organism age, condition, and sensitivity, water temperature, etc. If the laboratory does not have an ongoing culturing program and obtains the test organisms periodically in large numbers (batches) from an outside source, the sensitivity of each batch of test organisms must be evaluated with a reference toxicant within the seven days immediately preceding an effluent toxicity test or concurrently with the test. Laboratories that obtain test organisms from outside sources and conduct less than one toxicity test per month may find it convenient to run all sensitivity tests concurrently with effluent toxicity tests. If the laboratory maintains breeding stock, the sensitivity of the offspring should be checked with a reference toxicant at least once each month. If preferred, this reference toxicant test may be run concurrently with an effluent toxicity test.

CONTROL CHARTS

A control chart (USEPA, 1979a) should be prepared for each reference-toxicant/organism combination, and successive LC50s plotted and examined to determine if the results are within prescribed limits (Fig. 1). In this technique, a running plot is maintained for the LC50s from successive tests with a given reference toxicant. This type of control chart, called a cumulative-summation (cusum) chart, evaluates the cumulative trend of the statistics from a series of samples. The mean ($\bar{X}$) and upper and lower control limits ($\pm 2S$) are recalculated with each successive point, until the statistics stabilize. Outliers, which are values which fall outside the upper and lower control limits, and trends of increasing or decreasing sensitivity are readily identified.

If the LC50 of reference toxicant does not fall in the expected range for the test organisms, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects, and a different batch of test organisms should be employed in repeating the reference toxicant and effluent toxicity tests.

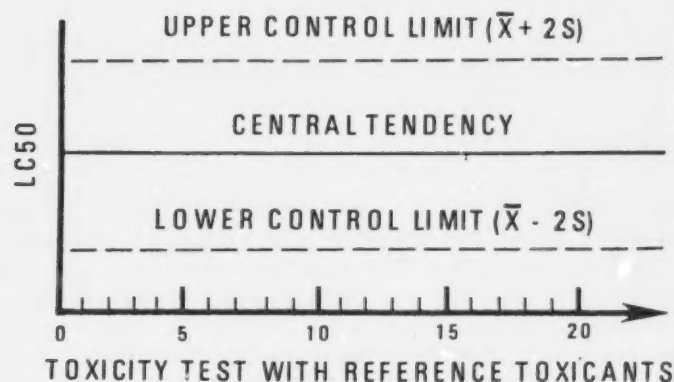


Figure 1. Control chart.

$$\bar{X} = \frac{\sum_{i=1}^n X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^n X_i^2 - \left(\sum_{i=1}^n X_i\right)^2/n}{n-1}}$$

Where:

X_i = Successive LC50's from toxicity tests.

n = Number of tests.

$\bar{X}$ = Mean LC50.

S = Standard deviation.

RECORD KEEPING

Proper record keeping is very important. Bound notebooks should be used to maintain detailed records of test organisms such as species, age, size, source, date of receipt, culture maintenance, and disease treatment, and information on the calibration of equipment, test conditions employed, and test results. Annotations should be made on a real-time basis to prevent the loss of information.

SECTION 4

FACILITIES AND EQUIPMENT

GENERAL REQUIREMENTS

Effluent toxicity tests may be performed in a fixed or mobile laboratory. Depending upon the scope of the toxicity screening program, facilities may include equipment for rearing, holding, and acclimating organisms. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, surface water, dechlorinated tap water, or synthetic water. It is recommended that receiving water be used for acclimation and testing wherever possible. Tap water can be dechlorinated by active aeration (using air stones) for 24 h, filtration through activated carbon, or the use of sodium thiosulfate. Aeration and treatment with sodium thiosulfate may not remove toxic halogenated organics produced by chlorination, whereas filtration with activated carbon will remove both the chlorine and the halogenated organics, and is the preferred treatment.

Equipment should be available for water temperature control and aeration. Air used for aeration must be free of oil and fumes. Test facilities must be well ventilated and free of fumes. During holding, acclimating, and testing, test organisms should be shielded from external disturbances.

Some organisms may have special environmental requirements such as flowing water, fluctuating water levels, or substrate, which must be provided. During holding, acclimating, and testing, immature stream insects should always be in flowing water (Nebeker and Lemke, 1968). Since cannibalism can occur among many species of shrimp, crabs and decapods, they should be isolated by some means (e.g., with screened compartments), or the claws of crabs and crayfish should be bound.

Materials used for dilutors, exposure chambers, tubing, etc., which come in contact with the effluent should be carefully chosen. Tempered glass, No. 304 (for freshwater) or No. 316 (or higher grade) stainless steel (for saline water), and perfluorocarbon plastics (e.g., TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Plastics such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used as test chambers and to store effluents and dilution water or to convey them to a test system. However, caution should be exercised in the use of plastics because they could introduce toxicants when new or carry over toxicants from one test to another. New plastics products of a type not previously used should be tested for toxicity before initial use. Small, inexpensive plasticware (except that of perfluorocarbon) which comes in contact with effluents should be discarded after each test. Equipment (large tanks, pumps, valves, etc.) which cannot

be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below. Fiberglass, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system. All materials should be flushed or rinsed with the test media for a period of time before using in the test.

Copper, galvanized material, rubber, brass, and lead, must not come in contact with holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers) may be toxic and should be tested before use.

EFFLUENT DILUTION SYSTEM (Flow-through test only)

Although many types of dilutors have been designed (Brungs and Lemke, 1978), the flow-through, proportional dilutor is the preferred system for routine effluent toxicity tests conducted in both fixed and mobile laboratories.

The following factors should be considered in designing the dilutor: (1) whether the apparatus will be installed and used in a fixed or mobile laboratory; (2) the existence of adequate space and/or structural requirements for the dilutor, test chambers, effluent and dilution-water storage; (3) the applicability of the dilutors to specific effluent characteristics (high suspended solids, volatiles, etc.); (4) the system's dependability, durability, flexibility, and ease of maintenance, cleaning, and replacement; (5) the ability to perform within the flow rate and concentration accuracy limitations; and (6) the cost of the system.

Until recently, it was necessary to custom-make all dilutors. However, prefabricated dilutor systems are now commercially available (e.g. Ace Glass¹; Specialized Environmental Equipment, Inc.²). Two examples of custom-made proportional dilutors -- the solenoid valve system and the vacuum siphon system -- are described in the Appendices.

TEST CHAMBERS

Type

The test chambers most commonly used in static tests for fish are wide-mouth, 0.95 L (1 qt), 3.8 L (1 gal) or 19.0 L (5 gal) soft-glass bottles or aquaria. Containers such as 10 to 20 cm (4 or 8 in.) diameter culture dishes or beakers may be more suitable as test chambers for fish eggs and/or larvae and small crustacea. Special glass or stainless steel test chambers can be constructed to accommodate test organisms requiring particular physical conditions (large surface-area-to-volume ratio).

¹Ace Glass Company, Vineland, New Jersey.

²Specialized Environmental Equipment, Inc., Easley, S.Carolina.

Test chambers used in flow-through tests are constructed of 3-mm (0.125-in.), double strength, or 6-mm (0.25-in.) tempered plate glass held together with clear silicone adhesive. Silicone adhesive absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water; extra beads of adhesive inside the containers should be removed. Stainless steel (No. 304 or No. 316) can be used in the construction of test chambers, but must be of welded, not soldered, construction.

In the event several species are to be tested simultaneously, the test chamber may be partitioned using dividers made from NITEX[®] 1, TEFLON[®], stainless steel, or glass. Also small cups made of NITEX[®], TEFLON[®] or glass may be used to segregate different species within the same test chamber. Commercially available test chambers may be used if they meet the above requirements.

The size of the chambers is varied according to the size of the test organism. All test chambers should be covered to prevent accidental contamination. The incidence of "floaters" (organisms trapped on the surface film) in Daphnia test chambers can be reduced by using opaque covers.

Cleaning

New plasticware used for sample collection or organism exposure vessels does not require rigorous cleaning. It is sufficient to rinse the new containers once with sample before use. New glassware, however, should be soaked overnight in acid (see Paragraph 3 below). It is also recommended that all sample containers, test vessels, and other equipment that has come in contact with effluent be washed after use in the manner described below to remove surface contaminants:

1. Soak 15 min, and scrub with detergent in tap water, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Rinse once with fresh, dilute (20%, V:V) nitric acid or hydrochloric acid (add 20 mL of concentrated acid to 80 mL of distilled water) to remove scale, metals, and bases.
4. Rinse twice with tap water.
5. Rinse once with full-strength, acetone to remove organic compounds.
6. Rinse well with tap water.
7. Rinse well with dilution water.

When feasible, the above cleaning procedure must also be used for other equipment that comes in contact with the effluent, such as the dilutor system, pumps, tanks, etc. All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

¹NITEX[®] is available from Tobler, Ernst, and Trabor, 2434 Dempster St., Des Plaines, Illinois 60016 (312-299-1051), or Tetco, Inc., 420 Sawmill River Rd., Elmsford, New York 10523, (914-592-5010).

SECTION 5

TEST ORGANISMS

SPECIES

The species used in characterizing the toxicity of an effluent will depend on the objectives of the test and the requirements of the regulatory agency. The organisms used in effluent toxicity tests must be identified to species. If there is any doubt as to the identity of the test organisms, they should be sent to a taxonomic expert for examination.

Species that have been widely used in toxicity tests and are acceptable test organisms are listed in Table 1. For practical reasons, the number of test species commonly used in effluent toxicity tests has been limited primarily to those organisms that are easily cultured in the laboratory.

Although it might seem appropriate to use marine organisms in toxicity tests of effluents discharged to saline waters, effluents generally consist of altered freshwater, and it would be appropriate to use freshwater organisms in the tests. The selection of freshwater and/or marine organisms for these tests may be dictated by site-specific conditions and/or may be specified in state water quality standards.

SOURCE

Although it might be desirable to use test organisms residing in the receiving water, it is usually not practical for the following reasons: (1) sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants; (2) it is often difficult to collect organisms of the desired age and condition from the receiving water; (3) most states require collection permits, which may be difficult to obtain. Therefore, it is usually more cost effective to obtain test organisms from private, state, and Federal sources. Care should be taken to ensure that test organisms are free of signs of stress and disease. Some species of test organisms, such as trout, can be obtained from "disease-free" stocks.

Some fish, such as the fathead minnow and silverside, and invertebrates, such as daphnids, midges, and mysid shrimp, are easily reared in the laboratory. Invertebrates and fish not amenable to laboratory rearing and not available from commercial or government sources, may be obtained in some cases by collecting them directly from wild populations. For methods of collecting fish and invertebrates see Weber (1973). However, test organisms obtained from the wild must be observed in

TABLE 1. RECOMMENDED SPECIES, TEST TEMPERATURES, AND LIFE STAGES.

Species	Test Temperature (°C) ^a	Life Stage ^b
<u>Freshwater</u>		
<u>Vertebrates</u>		
<u>Cold Water</u>		
Brook trout: <u>Salvelinus fontinalis</u>	12	30 - 90 days
Coho salmon: <u>Oncorhynchus kisutch</u>	12	30 - 90 "
Rainbow trout: <u>Salmo gairdneri</u>	12	30 - 90 "
<u>Warm Water</u>		
Bluegill: <u>Lepomis macrochirus</u>	20	1 - 90 days
Channel catfish: <u>Ictalurus punctatus</u>	20	"
Fathead minnow: <u>Pimephales promelas</u> ^c	20	"
<u>Invertebrates</u>		
<u>Cold Water</u>		
Stoneflies: <u>Pteronarcys</u> spp.	12	Larvae
Crayfish: <u>Pacifastacus leniusculus</u>	12	Juveniles
Mayflies: <u>Baetis</u> spp. or <u>Ephemere</u> lla spp.	12	Nymphs
<u>Warm Water</u>		
Amphipods: <u>Hyalella</u> , spp.,	20	Juveniles
<u>Gammarus lacustris</u> , <u>G. fasciatus</u> ,	20	"
or <u>G. pseudolimnaeus</u>	20	"
Cladocera: <u>Daphnia magna</u> or <u>D. pulex</u> , ^d	20	1 - 24 h
<u>Ceriodaphnia</u> spp.	20	1 - 24 h
Crayfish: <u>Orconectes</u> spp., <u>Cambarus</u> spp.,	20	Juveniles
<u>Procambarus</u> spp.,	20	"
Mayflies: <u>Hexagenia limbata</u> or <u>H. bilineata</u>	20	Nymphs
Midges: <u>Chironomus</u> spp.	20	Larvae

- To avoid unnecessary logistical problems in trying to maintain different test temperatures for each test organism, it would be sufficient to use one temperature (12°C) for cold water organisms and one temperature (20°C) for warm water organisms.
- The optimum life stage is not known for all test organisms.
- Mayes et al., 1983, found no significant difference in the sensitivity of fish ranging in age from 10 to 100 days, in tests with nine toxicants.
- Daphnia pulex is recommended over D. magna because it is more widely distributed in the United States, test results are less sensitive to feeding during tests, and it is not as easily trapped on the surface film.

TABLE 1. (CONTINUED)

Species		Test Temperature (°C)	Life Stage
<u>Marine and estuarine</u>			
<u>Vertebrates</u>			
<u>Cold Water</u>			
English sole:	<u>Parophrys vetulus</u>	12	1 - 90 days
Sanddab:	<u>Citharichthys stigmaeus</u>	12	"
Winter flounder:	<u>Pseudopleuronectes americanus</u>	12	Post-metamorphosis
<u>Warm Water</u>			
Flounder:	<u>Paralichthys dentatus</u>		
	<u>P. lethostigma</u>	20	1 - 90 days
Longnose killifish:	<u>Fundulus similis</u>	20	"
Mummichog:	<u>Fundulus heteroclitus</u>	20	"
Pinfish:	<u>Lagodon rhomboides</u>	20	"
Sheepshead minnow:	<u>Cyprinodon variegatus</u>	20	"
Silverside:	<u>Menidia spp.</u>	20	"
Spot:	<u>Leiostomus xanthurus</u>	20	"
Threespine stickleback:	<u>Gasterosteus aculeatus</u>	20	"
<u>Invertebrates</u>			
<u>Cold Water</u>			
Dungeness crab:	<u>Cancer magister</u>	12	Juvenile
Oceanic shrimp:	<u>Pandalus jordani</u>	12	"
Green sea urchin:	<u>Strongylocentrotus droebachiensis</u>	12	Gametes/embryo
Purple " " :	<u>S. purpuratus</u>	12	" "
Sand dollar:	<u>Dendraster excentricus</u>	12	" "
<u>Warm Water</u>			
Blue crab:	<u>Callinectes sapidus</u>	20	Juvenile
Mysid:	<u>Mysidopsis spp.</u>	20	1 - 5 days
	<u>Neomysis spp.</u>	20	" "
Grass shrimp:	<u>Palaemonetes spp.</u>	20	1 - 10 days
Penaid shrimp:	<u>Penaeus setiferus</u>	20	Post larval
	<u>P. duorarum</u>	20	" "
	<u>P. aztecus</u>	20	" "
Sand shrimp:	<u>Crangon spp.</u>	20	" "
Pacific oyster:	<u>Crassostrea gigas</u>	20	" "
American oyster:	<u>Crassostrea virginica</u>	20	Embryo/larval

the laboratory for one week, prior to use, to assure that they are free of disease and not suffering from stress due to capture and handling. Fish captured by electroshocking must not be used in toxicity testing.

LIFE STAGE

Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as first instars of daphnids and mysids, and juvenile fish, is recommended for all tests. There may be special cases, however, where the limited availability of organisms will require some deviation from the recommended life stage. In a given test, all organisms should be approximately the same age and should be taken from the same source. For the commonly used test organisms, the maximum difference in age within a batch is as follows: fathead minnow and silverside, ± 3 days; daphnids, ± 12 h; mysid, ± 24 h. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

LABORATORY CULTURING

Instructions for culturing fathead minnows, silversides, Daphnia, and mysids, are included in Appendix A.

HOLDING AND HANDLING

Except for new tanks, or where there is no previous history of disease problem, holding tanks or test chambers are disinfected before use with 0.5% commercial bleach for one hour (5 mL of bleach added to 1 L of water). Brush thoroughly with the disinfectant and rinse well between groups of organisms. Other equipment used to handle organisms must be disinfected with 0.5% commercial bleach. Note: Bleach is highly toxic. All vessels cleaned with bleach must be thoroughly rinsed with tap water, thiosulfate solution (50 mg/L), and then with uncontaminated holding water before the final filling and introduction of the test organisms.

To avoid unnecessary stress after collection, and during transportation and acclimation, organisms should not be subjected to changes of more than 30°C in water temperature or three parts per thousand (ppt) in salinity in any 12 h period.

Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk bolting cloth, plankton netting, or similar material. Nets coated with urethane resin are best for handling catfish. Wide-bore, smooth glass tubes (4 to 8 mm inside diameter) with rubber bulbs or pipettors (such as a Propipette^R or an electric pipettor) should be used for transferring smaller organisms such as daphnids, mysids, and larval fish.

Holding tanks are supplied with a good quality water (see Section 6) with a flow-through rate of at least two tank-volumes per day. Otherwise, use a recirculation system where the water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

When test organisms are obtained from a source known to have a healthy stock, a minimum observation period of 48 h is required. If the organisms are obtained from a source where the health of the stock is unknown, or obtained from the wild, a minimum observation time of seven days is required before use. The need for a lengthy observation period for organisms obtained from the wild may preclude the use of resident species in on-site toxicity tests.

Crowding should be avoided (see guidelines for loading, Section 8). The DO must be maintained at a minimum of 40% of saturation for warm water species and 60% of saturation for cold water species. Aeration is used if necessary.

Fish should be fed as much as they will eat at least once a day with live or frozen brine shrimp or dry food (frozen food should be completely thawed before use). Brine shrimp can be supplemented with commercially prepared food such as Tetramin^R flakes, BioRil^R, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning. Organisms should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. It is not uncommon to have some fish mortality in a holding tank because of individuals that refuse to feed on artificial food and, as a result, die of starvation.

A daily log of feeding, behavioral observations, and mortality should be maintained.

DISEASE TREATMENT

If fish are obtained from the wild, or from some source that is not certified as disease-free, it is recommended that prophylactic treatments be routinely utilized for external bacteria, trematodes, and protozoa. If fish are from inhouse cultures, or from sources certified as disease-free, therapeutic treatments should be used only when disease is observed. If the disease is not serious and responds to treatment, the fish may be used after a disease-free period of seven days.

Recommended prophylactic and therapeutic treatments for freshwater fish are listed in Table 2. Some of these chemicals can also be used to treat saltwater fish. When fish are severely diseased, the entire lot should be discarded. Invertebrates which become diseased should be discarded. Tanks which are contaminated with disease-causing microorganisms must be disinfected with 0.5% commercial bleach and thoroughly rinsed (see p. 16).

TABLE 2. RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR DISEASE IN FRESHWATER FISH^{a,b}

Disease	Chemical	Concentration ^c (mg/L)	Duration of Treatment
External bacteria	Terramycin (oxytetracycline hydrochloride, water soluble)	25 ^c	30-60 min
	Penicillin G Procaine ^d (In dihydrostreptomycine Sulfate Solution)	(3mL/100gal)	48-72 h
	Benzalkonium chloride (Hyamine 1622 ^R or 3500 ^R)	1-2 ^c	30-60 min
	Furanace	0.05 - 0.1 1.0	Indefinite 5-10 min

Monogenetic trematodes fungi, and external protozoa ^e	Formalin	250	up to 60 min
	Potassium permanganate	2-6	30-60 min
	Sodium chloride	15,000-30,000 2,000-4,000	5-10 min dip 24 h minimum

Parasitic copepods	Trichlorfon (Masoten ^R)	0.25 ^c	Continuous ^f

a. This table indicates the order of preference of treatments that have been reported to be effective, but their efficacy against disease and toxicity to fish may be altered by species, temperature or water quality. CAUTION: TREATMENTS SHOULD BE TESTED ON SMALL LOTS OF FISH BEFORE MAKING LARGE-SCALE APPLICATIONS. Additional information may be obtained from sources such as Davis (1953), Hoffman and Meyer (1974), Reichenbach-Klinke and Elkan (1965), Sniewzko (1970), van Duijn (1973), and Herwig (1979).

TABLE 2. Footnotes (Continued)

- b. Treatment may be accomplished by: (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical; or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Brungs and Mount (1967).
- c. Active ingredient.
- d. Treatment of fish with Penicillin G Procaine immediately after they are brought into the lab may be necessary to prevent outbreaks of bacterial infections caused by stress from handling. Penicillin can be obtained from veterinarians, and from animal feed and supply stores.
- e. Frequently, treatments for trematodes and protozoans need to be continued for three successive days. Ichthyophthirius ("Ich") must be treated daily or every other day until no sign of the protozoan remains.
- f. Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for more than four weeks and should not be used above 27°C.

TRANSPORTATION TO THE TEST SITE AND ACCLIMATION TO DILUTION WATER

When organisms are to be transported from the base laboratory to a remote test site, it is convenient to move them in plastic bags placed in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags. Another method commonly used to maintain sufficient DO during shipment is to aerate with an airstone which is supplied from a portable pump. During transport and acclimation, the organisms should not be subjected to a change of more than 3°C in water temperature or 3 ppt salinity in 12 h. The DO concentration must not fall below 40% of saturation for warm water species and 60% of saturation for cold water species.

Upon arriving at the test site, the organisms are acclimated to the test dilution water and temperature by changing from holding water to dilution water. All but a small volume (approximately 5%) of the holding water is removed by siphoning, and replaced slowly over a 10 to 15 min. period with dilution water. If receiving water is used as dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only 50% of the test organisms be exposed initially to the dilution water. If the mortality does not exceed 5% in 24 h, the remainder of the test organisms are acclimated to the dilution water. All organisms must be exposed to 100% dilution water, within the temperature range of the test, for a minimum of 24 h before they are used in the effluent toxicity tests.

A group of organisms must not be used for a test if they appear to be diseased, discolored, or otherwise stressed (Sprague, 1969), or if more than 5% die during the 24- to 48-h period immediately preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. As stated above, the mortality may be due to the presence of toxicity in the dilution (receiving) water, rather than a diseased condition of the test organisms. If the acclimation period is repeated with a new group of test organisms and mortality exceeds 10%, it is recommended that an alternative source of dilution water be used.

The acclimation of marine organisms for effluent toxicity tests poses special problems because most effluents discharged into the marine environment consist of altered freshwater. Therefore, when the effluent is diluted with the receiving water (salt water), the higher percent effluent volumes will have a low salinity (the salinity will be inversely proportional to the percent volume of effluent). If the effluent is essentially freshwater, it is obvious that 100% effluent cannot be used in a flow-through system with marine test organisms. In this case, the test is conducted without the inclusion of 100% effluent as one of the treatments. However, depending upon the objectives of the test and the policy of the regulatory agency, the problem of salinity control may be avoided altogether by using a freshwater test species and freshwater as the diluent.

In static tests, marine organisms can be used at all concentrations of effluent by adjusting the salinity of the effluent to a standard salinity (such as 20 ppt) or to the salinity of the receiving water, by adding sufficient dry ocean salts, such as Instant Ocean (or equivalent)¹. Saline dilution water can also be prepared by adding dry salts to the receiving water or to distilled water or a freshwater such as well water or a suitable surface water. Care must be taken to ensure that the added salts are completely dissolved before the test organisms are placed in the solutions (see discussion in Preparation of Test Solutions, Section 8). For tests in which dry ocean salts are used to obtain the desired salinity, the test organisms should be acclimated in synthetic saline water prepared with the dry salts. However, addition of dry ocean salts to dilution water may result in an increase in pH. It may be desirable to lower the pH of the salinity-adjusted effluent, before using it in the test, by the addition of (1N) hydrochloric acid.

If it is not possible to maintain the same salinity in all effluent concentrations because of the large volumes of water required, such as in flow-through tests, the highest effluent concentration (lowest salinity) that could be tested would depend upon the salinity of the receiving water and the tolerance of the test organisms. The tidewater silverside and mysid, for example, are known to tolerate a salinity range of 5 to 35 ppt, and the inland silverside has a salinity tolerance range of 0 to 30 ppt. However, the tolerances of other marine species would have to be determined by the investigator in advance of the test.

Because of the circumstances described above, when performing flow-through tests of effluents discharged to saline waters, it is advisable to acclimate groups of test organisms to each of three different salinities, such as 10, 20, and 30 ppt, prior to transporting them to the test site (see p. 20). It would also be advisable to maintain cultures of these test organisms at a series of salinity levels, including at least 10, 20, and 30 ppt, so that the change in salinity upon acclimation at the desired test dilutions does not exceed 6 ppt.

It may be desirable to conduct static tests at several salinities as described above.

¹Available dry ocean salts, and sources of supply include:

- (a) Instant Ocean^R. Aquarium Systems, 8141 Tyler Blvd., Mentor, Ohio 44060 (216-255-1997).
- (b) Marine Environment^R. Import Associates, Inc., P.O. Box 16350, San Francisco, California, 94116 (415-591-2200).
- (c) h.w. Marine Mix^R. Hawaiian Marine Import, Inc., P.O. Box 218687, Houston, Texas 77218 (713-492-7864).
- (d) Forty Fathoms^R. Marine Enterprises, Inc., 8755 Mylander Lane, Towson, Maryland, 21204 (301-321-1189).
- (e) Jungle Ocean 50^R. Jungle Laboratories Corp., Box 630, Cibolo, Texas, 78108 (800-327-2200, or 512-658-3503).
- (f) Rila Marine Mix^R. Rila Products, P.O. Box 114, Teaneck, New Jersey, 07666 (201-836-0855).
- (g) Kahl Sea Salt^R. Kahl Scientific Instrument Corporation, P.O. Box 1166, El Cajon, California, 92202-1166 (619-444-2158 or 5944).

SECTION 6

DILUTION WATER

Dilution water used in the tests will depend on the objectives of the study and other factors. Receiving water collected upstream from the discharge point should be used as dilution water wherever possible. Depending upon the objectives, it may be desirable to use receiving water as the dilution water even when it is contaminated by "upstream" sources; e.g. if the objective of the test is to determine the effect of multiple point sources. However, if because of the study objectives the presence of contaminants in the receiving water makes it unsuitable as dilution water, or it is not economically feasible to collect and ship receiving water to a remote laboratory for off-site testing, other surface waters such as nearby tributaries, or ground water, or synthetic water may be employed.

When the dilution water is taken from the receiving water, it should be collected at a point as close as possible to the outfall, but upstream from or outside of the zone influenced by the effluent. The water used to make up the effluent dilutions should be collected immediately prior to the test, but never more than 96 h before the test begins.

If contaminant-free water is required in the test, dilution water is acceptable if healthy test organisms survive in it without signs of stress and mortality does not exceed 5% during the acclimation period.

Recommended procedures for the preparation of synthetic freshwater and sea water are given in Tables 3 and 4, respectively. A more convenient method of preparing synthetic salt water is to add commercially available dried ocean salts (see Section 5) to distilled or de-ionized water. However, addition of dry ocean salts to dilution water may result in an increase in pH. If this occurs, the pH of the salinity-adjusted water should be lowered to the desired level for the test by the addition of (1N) hydrochloric acid.

In an estuarine environment, the investigator should collect uncontaminated water having a salinity as near as possible to the salinity of the water at the receiving site. Water should be collected at slack high tide, or within one hour after high tide. If there is reason to suspect contamination of the water in the estuary, it is advisable to collect uncontaminated water from an adjacent estuary. At times it may be necessary to collect water at a location closer to the open sea, where the salinity is relatively high. In such cases, uncontaminated freshwater is added to the saline water to dilute it to the required test salinity.

When necessary, the dilution water should be pretreated by filtering through a nylon sieve having 2- to 4-mm holes to remove debris and/or break up large floating or suspended solids. If a mini-dilutor employing capillary delivery tubes is used, it may be necessary to filter the effluent through glass wool prior to entering the dilutor system.

It is preferable to pump the dilution water continuously to the acclimatic tank and dilutor. However, it may be more practical to transport batches of water to the testing site in tanks, and continuously pump water from the tanks to the acclimation chamber and dilutor.

TABLE 3. PREPARATION OF SYNTHETIC FRESH WATER^a.

Water Type	Reagent Added (mg/L) ^b				Final Water Quality		
	NaHCO ₃	CaSO ₄ ·2H ₂ O	MgSO ₄	KCl	pH ^c	Hardness ^d	Alkalinity
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

a. Taken in part from Marking and Dawson (1973).

b. Add reagent grade chemicals to distilled or deionized water

c. Approximate equilibrium pH after aerating 24 h.

d. Expressed in mg/L as CaCO₃.

TABLE 4. PREPARATION OF SYNTHETIC SEA WATER.^{a,b,c}

Chemical	Amount
NaF	3 mg
SrCl ₂ ·6H ₂ O	20 mg
H ₃ BO ₃	30 mg
KBr	100 mg
KCl	700 mg
CaCl ₂ ·2H ₂ O	1.47 g
Na ₂ SO ₄	4.00 g
MgCl ₂ ·6H ₂ O	10.78 g
NaCl	23.50 g
Na ₂ SiO ₃ ·9H ₂ O	20 mg
Na ₄ EDTA ^d	1 mg
NaHCO ₃	200 mg

- Add reagent-grade chemicals in the order listed, to 890 mL distilled or deionized water. Each chemical must be dissolved before another is added.
- The final desired test salinity is attained by dilution at the time of use. If the above solution is diluted to one liter, the salinity should be 34 ± 0.5 ppt and the pH 8.0 ± 0.2 .
- From Kester et al. (1967), Zarogian et al. (1973), and Zillioux et al. (1973).
- Tetrasodium ethylenediaminetetraacetate. This compound should be omitted when toxicity tests are conducted with metals. When tests are conducted with plankton or larvae, the EDTA should be omitted and the medium should be stripped of trace metals (Davey et al., 1970).

SECTION 7

EFFLUENT SAMPLING AND HANDLING

EFFLUENT SAMPLING

Ordinarily, the effluent sampling point is the same as that specified in the NPDES discharge permit (USEPA, 1979c), which is usually the point of discharge (outfall). Conditions for exception would be: (1) if there is better access to a sampling point between the final treatment and the discharge point; (2) if the processed waste is chlorinated prior to discharge to the receiving waters, the sampling point may be located prior to contact with chlorine if the purpose of the test is to determine toxicity levels of the unchlorinated effluent; or (3) if there is a desire to evaluate separate wastewater streams prior to their being combined with other wastewater streams, or noncontact cooling water. Some regulatory authorities measure the toxicity of the effluent prior to chlorination, after chlorination, and after dechlorination with sodium thiosulfate.

At times it may be necessary to filter the effluent through glass wool or similar material to remove solids which would clog the orifices of solenoid valves and capillary tubes used in flow-through systems. Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the toxicity of the effluent is variable, grab samples collected during peaks of effluent toxicity provide a measure of maximum effect. The compositing process has an averaging effect which tends to dilute the toxicity peaks, and may provide misleading results when testing for acute toxicity. Composited samples, therefore, are more appropriate for chronic toxicity tests where peak toxicity of short duration is of less concern (USEPA, 1984b).

SAMPLE TYPE

The advantages and disadvantages of grab and composite samples are listed below:

1. Grab Samples

a. Advantages:

- (1) Easy to collect; require a minimum of equipment and on-site time.
- (2) Provide a measure of instantaneous toxicity. Toxicity spikes are not masked by dilution.

b. Disadvantages:

- (1) Samples are collected over a very short period of time and on a relatively infrequent basis. The chances of detecting a spike in toxicity would depend on the frequency of sampling.
- (2) A separate test must be conducted with each grab sample, if they are to be useful.

2. Composite Samples:

a. Advantages:

- (1) A single effluent sample is collected over a 24-h period, and only one toxicity test is conducted.
- (2) The sample is collected over a much longer period of time and contains all toxicity spikes.

b. Disadvantages:

- (1) Sampling equipment is more sophisticated and expensive, and must be placed on-site for at least 24 h.
- (2) Toxicity spikes may not be detected because they are masked by dilution with less toxic wastes.

TEST TYPE

1. Static Tests

Sampling recommendations are discussed for two types of test -- nonrenewal and renewal. For nonrenewal tests, effluent samples are collected only at the beginning of the test. For renewal tests, samples are generally collected to renew the test solutions daily throughout the test period.

The following effluent sampling methods are recommended for static tests:

a. Continuous Discharges

- (1) If the facility discharge is continuous, but the calculated retention time of a continuously discharged effluent is less than 14 days and the variability of the waste is unknown, one of the following approaches is used:
 - (a) Nonrenewal tests: a minimum of four separate grab samples are collected at evenly-spaced (6-h) intervals over the first 24-h period and used in four separate tests to determine the variability in toxicity.
 - (b) Renewal tests: a minimum of four separate grab samples are collected at evenly-spaced (6-h) intervals over the first 24-h period and used in four separate tests begun on the first day and renewed daily with samples collected at the appropriate time.

- (2) If the calculated retention time of a continuously discharged effluent is greater than 14 days, or if it can be demonstrated that the wastewater does not vary in chemical composition or concentration regardless of holding time, one of the following approaches is used:
 - (a) Nonrenewal tests: a minimum of one grab sample is collected and used in a single test.
 - (b) Renewal tests: a minimum of one grab sample is collected each day and used to renew the test solutions.

The retention time of the effluent in the wastewater treatment facility may be estimated from calculations based on the volume of the retention basin and rate of wastewater inflow. However, the calculated retention time may be much greater than the actual time because of short-circuiting in the holding basin. Where short circuiting is suspected, or sedimentation may have reduced holding basin capacity, a more accurate estimate of the retention time can be obtained by carrying out a dye study.

b. Intermittent Discharges

If the facility discharge is intermittent, one of the following approaches is used:

- (1) Where the effluent is continuously discharged during a single eight-hour work shift or two successive eight-hour work shifts, a minimum of one grab sample is collected midway during the discharge period and used in a single nonrenewal test, or a grab sample may be collected daily for a renewal test.
- (2) Where the facility retains the wastewater during an eight-hour work shift, then treats and releases the wastewater as a batch discharge, a grab sample is collected for a single nonrenewal test, or a grab sample is collected daily for a renewal test.
- (3) Where the facility discharges wastewater to an estuary only during an outgoing tide (usually during the four hours following slack high tide), a grab sample is collected during a discharge period for use in a single nonrenewal test, or a grab sample is collected daily for a renewal test.
- (4) At the end of the shift, clean up activities may result in the discharge of a slug of toxic waste. It would be advisable, therefore, to consider collecting a sample at that time and conducting a separate toxicity test.

2. Flow-through Tests

The following effluent sampling methods are recommended for flow-through tests:

a. Continuous Discharges

If the facility discharges continuously, the effluent should be pumped directly and continuously from the discharge line to the dilutor system for the duration of the test. However, if the effluent cannot be pumped directly and continuously to the dilutor system, the following alternative methods are employed for collection of the effluent:

- (1) Where the calculated retention time of the effluent is less than 14 days, two grab samples are collected daily (e.g., 8:00 AM and 4:00 PM). The freshly collected effluent should not be combined with the effluent remaining from the previously collected sample. The remaining part of the previously collected sample is discarded and the container is refilled with the fresh effluent.
- (2) Where the calculated retention time of the effluent is 14 days or greater, a single grab sample of sufficient volume to supply the dilutor for 24 h is collected daily. Here again, the volume of sample remaining from the previous day is discarded and replaced by the fresh sample.

b. Intermittent Discharges

If the facility discharge is intermittent, one of the following procedures is used:

- (1) Where a continuous discharge occurs during a single eight-hour work shift or two successive eight-hour work shifts, at least one grab sample of sufficient volume to supply the dilutor for 24 h is collected daily, midway during the discharge period.
- (2) Where the facility retains the wastewater during an eight-hour work shift, and then treats and releases it in a batch discharge, a single grab sample of sufficient volume to last 24 h is collected daily during the test period.
- (3) Where the facility discharges wastewater to an estuary only during an outgoing tide (usually during the four hours following slack high tide), a single grab sample of sufficient volume to last 24 h is collected during one discharge period every 24 h for the duration of the test. An alternate sampling method would be to place the effluent sampling pump in the final waste lagoon adjacent to the discharge pipe so that a continuous source of effluent would be available for the test.

EFFLUENT HANDLING

1. Samples Used in On-site Tests

a. Flow-through tests

Effluent grab samples used in on-site, flow-through toxicity tests should be stored in covered, unsealed containers. Stainless steel or fiberglass tanks are preferred because they can be easily decontaminated and reused with different effluents. Because of the large volume of sample normally required for flow-through tests with fish -- approximately 400 L (100 gal)/day -- the samples are usually held outside the laboratory at ambient air temperatures. It is not feasible to cool the sample prior to its transfer from the sample holding tank through the temperature control equipment and to the dilutor system. However, in cold weather, it may be necessary to use heater tape wound around the incoming line, or agitation with a submersible pump, to prevent freezing.

b. Static renewal and nonrenewal tests

Samples that are collected for on-site tests should be used within 24 h. Samples that are not used immediately should be iced or refrigerated.

2. Samples Shipped to Off-Site Test Facility

Samples collected for off-site toxicity testing are to be immediately placed on ice, and are shipped iced to the central laboratory, where they are transferred to a refrigerator (4°C) until used. Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory. The maximum lapsed time from collection of a grab or composite sample and the initiation of the test should not exceed 72 h. However, if the persistence of the toxicity of the sample is unknown, the maximum holding time should not exceed 36 h.

Nonrenewal static tests generally require only 4 to 20 L (one to five gallons) of effluent, which can be shipped in 4-L (1-gal) glass jugs, CUBITAINERS[®], or plastic "milk" jugs. Glass jugs can be cleaned and reused. CUBITAINERS[®] and plastic jugs are punctured and discarded after use.

SECTION 8

TOXICITY TEST PROCEDURES

RANGE-FINDING TEST

EPA Regional and state personnel generally have observed that it is not necessary to conduct a range-finding test prior to initiating a static, acute, definitive toxicity test. However, when flow-through tests are used, it is advisable to conduct a preliminary range-finding test because of the lack of flexibility in changing the concentration after the test is underway. The range-finding test most often used is an abbreviated static test in which groups of five organisms are exposed to three to five widely spaced effluent dilutions, such as 1%, 10%, 50%, and a control, for eight to 24 h (for information on other test conditions, see the Definitive Test Section below).

It should be noted that the toxicity (LC50) observed in range-finding tests may be significantly different from the toxicity observed in the definitive test because: (1) the definitive test is longer; and (2) the test is performed with an effluent sample collected at a different time, and the characteristics of the effluent and the receiving water may vary significantly within short periods of time.

SCREENING TEST

Some Regional and State effluent biomonitoring programs stipulate the use of abbreviated tests with a single (100%) effluent concentration for acute toxicity screening purposes (Weber and Peltier, 1981). The duration of the screening test is usually 24 h. However, where effluents are acutely toxic, mortality often occurs within the first few hours of exposure. In such cases, the test can be terminated within six to eight hours. If lethality is observed in the screening test, a definitive test may be required.

Acute toxicity screening tests are carried out as follows:

1. Measure the pH and DO of the (100%) effluent and control water. If the pH falls outside the range of 6.0 - 9.0, set up two parallel tests, using pH-adjusted (pH = 7.0) and unadjusted 100% effluent. The effluent sample is adjusted to pH 7 by adding 1.27 mL 1N NaOH and 2.0 mL 1M KH_2PO_4 per litre (Marking and Dawson, 1973). If the DO is less than 40% of saturation when using warm water species, or less than 60% when using cold water species, the test solutions should be aerated before use.
2. For each test, a total of 20 organisms are distributed among one to four test vessels containing 100% effluent.

3. A total of 20 organisms are also immediately distributed among one to four vessels containing control medium consisting of reconstituted water.

4. The test vessels are checked frequently for mortality during the first few hours, and the following action is taken:

- a. If the mortality of the organisms exposed to 100% effluent exceeds 10% at any time during the first few hours, and the mortality in the control has not exceeded 10%, the effluent is considered to exhibit acute toxicity. The screening test is immediately terminated, and a definitive test is initiated.
- b. If the mortality of the organisms in the control beakers exceeds 10% at any time during the first few hours or at any time later in the test, the preliminary screening test is immediately terminated and repeated.
- c. If the mortality in the effluent and control vessels is less than 10% after the first few hours, the test is continued until 10% mortality is exceeded in either the effluent or controls, or until the test period reaches 24 h, whichever occurs first.

5. If the test runs for the full 24-h period, the results are interpreted as follows:

- a. If the mortality in the effluent sample was less than 10%, the effluent sample is not considered to be acutely toxic, and no further acute tests are conducted on that sample unless the mortality in the controls exceeded 10% or the reference toxicant data were not acceptable.
- b. If the mortality exceeded 10% in the effluent sample, but was less than 10% in the controls, the effluent sample is considered to exhibit acute toxicity and a definitive test is conducted (see below).
- c. If the mortality exceeded 10% in the controls, or if the reference toxicant data were not acceptable, the screening test must be repeated.

DEFINITIVE TEST

"Definitive" tests are distinguished from "screening" tests primarily by: (1) the greater length of the test period; and (2) the use of multiple concentrations of effluent to obtain a point estimate of toxicity in terms of a LC50. Flow-through tests have the advantage of detecting temporal changes in effluent toxicity, and the longer exposure period of the definitive test increases the probability that the test period will include toxicity spikes, if they occur.

1. Test Duration

See the Summary of Test Conditions for information on test duration. In static tests, most of the mortality generally occurs during the first 24 to 48 h. Therefore, there may be little advantage to extending these tests to 96 h. However, state water quality standards may require a 96-h test period.

2. Preparation of Test Solutions

The definitive toxicity test includes a control and a series of effluent concentrations. The effluent concentrations are commonly selected to approximate a geometric series, such as 100%, 50%, 25%, 12.5%, and 6.25%.

a. Static Tests

An excess volume (e.g., 8 L) of each solution is prepared to provide sufficient material for toxicity testing and chemical analysis. The solutions are well mixed with a glass rod, TEFLON^R stir bar or other means. The DO is checked, and the solutions are aerated if necessary to raise the DO concentration to the required level. Aliquots of each concentration are delivered to the test chambers, and the chambers are arranged in random order. The chambers are placed in a water bath or otherwise brought to the required temperature, and the test organisms are added as described in Paragraph 4. The remaining volumes of each effluent concentration can be used for the chemical analyses.

Saline dilution water can be prepared by adding dry salts to the receiving water, or to distilled or de-ionized water, or a freshwater such as well water or a suitable surface water to obtain the same salinity as the effluent (see p. 21). If receiving water (saline water) is used as the diluent, a salinity control must be prepared using distilled or deionized water and dried sea salts to determine if the addition of sea salts alone has an adverse effect on the test organisms. As noted above, it may be desirable to conduct static toxicity tests at several salinities.

If the effluent is freshwater and the test is to be conducted with a salt water organism, depending on the objectives of the test and the policy of the regulatory agency, the test solutions may be prepared by adding dried ocean salts (see Section 6) to a sufficient quantity of 100% effluent to raise the salinity to the desired level (usually 20 ppt). After the addition of the dried salts, stir gently for 30 to 60 min, preferably with a magnetic stirrer, to ensure that the salts are in solution. It is important to check the final salinity with a refractometer or salinometer.

Addition of dry ocean salts to effluents and dilution water may change the pH and affect the toxicity of the waste. The pH of the salinity-adjusted solutions can be brought to the desired level by addition of acid or base (1N HCl or NaOH). However, it is recommended

that a concurrent test be conducted with salinity-adjusted effluent in which the pH has not been altered after adding the salt.

The volume of the effluent used must be sufficient to prepare all percent concentrations of the effluent needed for the toxicity test and for chemical analysis. For example, to conduct tests with Menidia, the use of 500 mL of test solution in each of duplicate exposure vessels and five concentrations of effluent (10 exposure vessels), would require a total of 2 L of 100% effluent. However, to provide sufficient volumes of test solutions for chemical analysis and for toxicity testing with invertebrates, additional effluent would be required.

Note: If after one hour, 100% mortality has occurred in the higher effluent concentrations (100% and 50%), additional concentrations of effluents, such as 3.1%, 1.5%, and 0.75%, are added to the test at the lower end of the concentration series.

b. Flow-through Tests

Flow-through tests are usually performed with the same effluent concentrations that are used for the static tests, except that where the receiving water is saline and the effluent is not, 100% effluent can not be tested with a marine organism.

The dilutor system should be operated 24 h prior to adding the test organisms. During this period, the dilutor is calibrated and the necessary adjustments can be made in the temperature, flow rate through the test chambers, and aeration. The flow rate through the proportional dilutor must provide for a minimum of five 90% replacements of water volume in each test chamber every 24 h (see Fig. 2). This replacement rate should provide sufficient flow to maintain an adequate concentration of dissolved oxygen. The dilutor should also be capable of maintaining the test concentration at each dilution within 5% of the starting concentration for the duration of the test. The calibration of the dilutor should be checked carefully before the test begins. This check should determine the volume of effluent and dilution water used in each portion of the effluent delivery system and the flow rate through each test chamber. The general operation of the dilutor should be checked at least at the beginning and end of each day during the test.

3. Control

The control consists of the same dilution water, test conditions, procedures, and organisms used in testing the effluent. In the event a test is to be conducted with salt water organisms, where each effluent dilution has a different salinity, a static control is prepared for the lowest (or highest, in the case of high salinity, e.g. brine wastes) salinity level used in the flow-through test to determine if salinity alone has any adverse effects on the test organisms. For a valid test, control mortality must not exceed 10%.

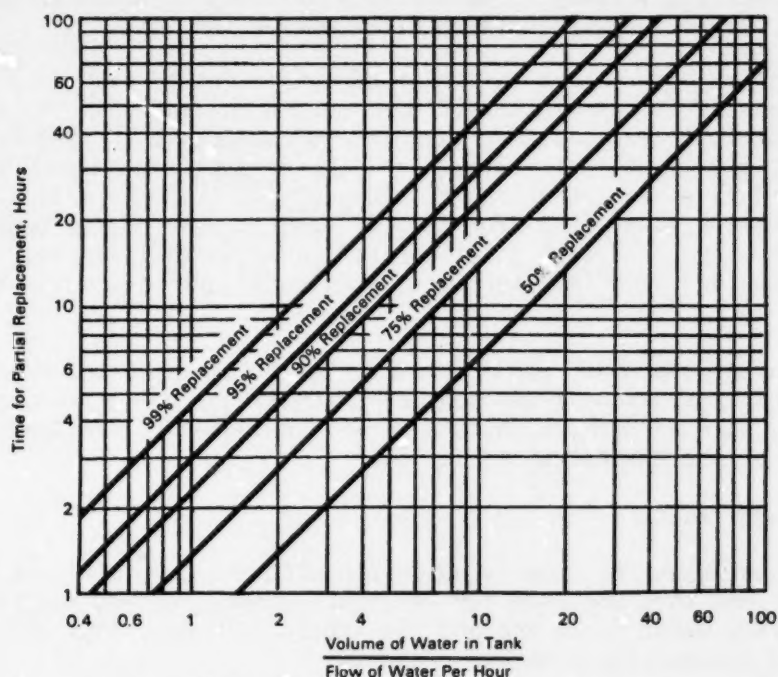


Figure 2. Approximate times required to replace water in test chambers in flow-through tests. For example: for a chamber containing 4 L, with a flow of 2 L/h, the above graph indicates that 90% of the water would be replaced every 4.8 h. The same time period (such as hours) must be used on both axes, and the same unit of volume (such as litres) must be used for both volume and flow. (From: Sprague, 1969).

4. Number of Test Organisms

At least 20 organisms of a given species are exposed to each effluent concentration (Jensen, 1972). More than one species may be used in the same test chamber in a given test, if segregated. Small fish and invertebrates are captured with 4- to 8-mm inside diameter pipettes. Organisms larger than 5 mm are captured by dip net. In a typical toxicity test involving five effluent concentrations and a control (six concentrations X 20 organisms/concentration), fish and other large test organisms are captured from a common pool and distributed sequentially to the test chambers until the required number of organisms are placed in each. Some small organisms, such as daphnids, mysids, and larval fish, are first distributed to small intermediary holding vessels, such as weighing boats, petri dishes, or small beakers. The water in the intermediary holding vessels is then drawn down to a small volume and the entire lot is transferred to a test chamber.

5. Replicate Test Chambers

One or more test vessels are provided for each effluent concentration and the control. In the past, it has been common practice in toxicity tests to require duplicate test vessels for each effluent concentration and control. However, these duplicates were usually not prepared independently, and thus were not true replicates. Also, the data from duplicate chambers are usually combined to determine the LC50 and confidence interval. Since it is not essential, statistically, to maintain two or more replicate test chambers at each effluent concentration to determine the LC50 and its confidence interval, the practice of dividing the test population for each effluent concentration between two or more replicate chambers must be considered optional. The practice has several advantages, however, and is encouraged because it: (1) permits easier viewing and counting of test organisms; (2) more easily avoids possible violation of loading limits, which might occur if all (20) of the test organisms are placed in a single test vessel; and (3) ensures against the invalidation of the test which might result from accidental loss of a test vessel, where all of the test organisms for a given treatment are in a single chamber.

6. Loading of Test Organisms

A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the metabolic conversion of significant amounts of effluent constituents, the accumulation of injurious concentrations of metabolic waste products, and/or stress induced by crowding, any of which could significantly affect the test results.

For flow-through tests, the live weight of test organisms in the test chambers must not exceed 5 g/L of test solution at temperatures of 20°C or less and minimum exchange rate (see above), or 2.5 g/L of test solution at temperatures above 20°C.

For both renewal and non-renewal static tests, loading in the test chambers must not exceed 0.8 g/L of test solution at temperatures of 20°C or less and 0.4 g/L of test solution at temperatures above 20°C.

7. Test Temperature

The temperature of the test solutions must be maintained within $\pm 2.0^\circ\text{C}$ of the recommended temperature for the test organism (Table 1). This can be accomplished for flow-through tests by passing the effluent and/or dilution water through separate coils immersed in a heating or cooling water bath prior to entering the dilutor system. Coils should be made from materials recommended in Section 4. For static tests, the temperature can be controlled by use of a water bath, room air conditioner, or environmental chamber.

8. Stress

Minimize stress on test organisms by avoiding unnecessary disturbances.

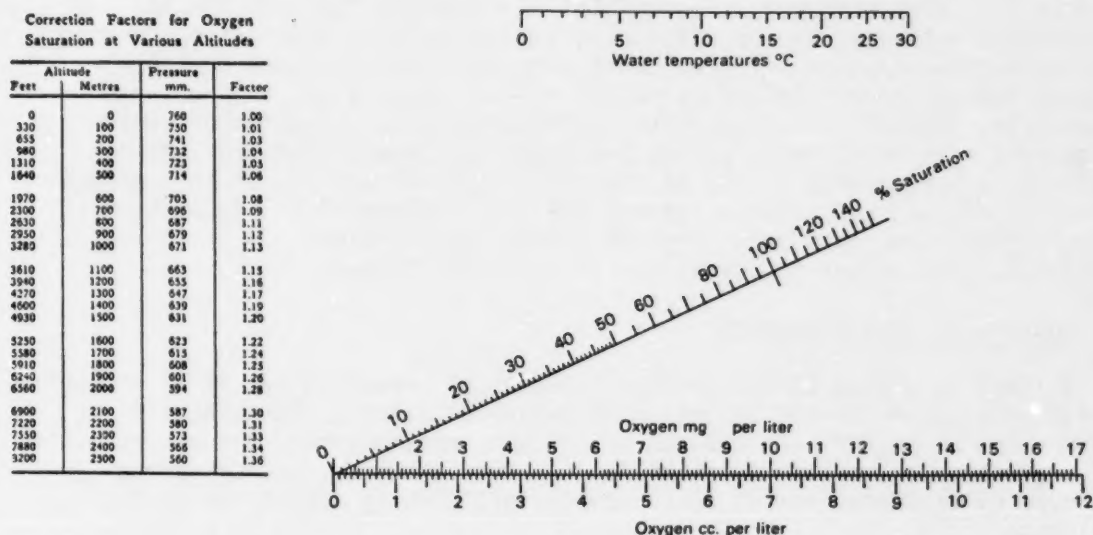


Figure 3. Rawson's nomogram for obtaining oxygen saturation values at different temperatures at sea level. When a straightedge is used to connect the water temperature on the upper scale and the concentration on the lower scale, the percent saturation can be read from the point of intersection on the diagonal scale. To determine the percent saturation at locations above sea level, factors are provided to convert oxygen concentrations measured at various altitudes to sea level values in the table at the upper left. For example, an oxygen concentration of 6.4 mg/L measured in a body of water at an altitude of 1000 m at a temperature of 15°C would be equivalent to a concentration of 6.4×1.13 , or 7.2 mg/L at sea level. To determine the percent saturation, a straight edge is used to connect the point at 15°C on the temperature scale with the point, 7.2 mg/L on the concentration scale, and the percent saturation is read at the point of intersection (68%) on the diagonal scale. (From Welch, 1948).

9. Dissolved Oxygen Concentration

Aeration may alter the results of toxicity tests and should be used only as a last resort to maintain the required DO. Aeration can reduce the apparent toxicity of an effluent by stripping it of highly volatile toxic substances, or increase its toxicity by altering the pH. However, the DO in the test solution must not be permitted to fall below 40% of saturation for warm water species and 60% of saturation for cold water species (Fig. 3).

In most flow-through tests, DO depletion is not a problem in the test chambers because aeration occurs as the liquids pass through the dilutor system. If the DO decreases to a level that would be a source of additional stress, the turnover rate of the solutions in the test chambers must be increased sufficiently to maintain acceptable DO levels. If the increased turnover rate does not maintain adequate DO levels, aerate the dilution water prior to the addition of the effluent, and aerate all test solutions. To reduce the potential for driving off volatile compounds in the wastewater, aeration may be accomplished by bubbling air through a 1-ml pipet at a rate of no more than 100 bubbles/min, using an air valve to control the flow. The turbulence caused by aeration should not result in a physical stress to the test organisms.

In static tests, low DOs commonly occur in the higher concentrations of wastewater. Aeration is accomplished by bubbling air through a pipet as described above. It is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test.

Caution must be exercised to avoid excessive aeration. When aeration is used, the methodology must be detailed in the report. For safety reasons, pure oxygen should not be used to aerate test solutions.

10. Illumination

Light of the duration, quality, and intensity normally obtained in the laboratory during working hours is adequate (see Summary of Test Conditions, pp. 39-42).

11. Feeding

a. Static Tests

Problems caused by feeding, such as the possible alteration of the toxicant concentration, the build-up of food and metabolic wastes and resulting oxygen demand, are common in static test systems. Where feeding is necessary, however, excess food should be removed daily by aspirating with a pipette.

b. Flow-through Tests

Feeding does not cause the above problems in flow-through systems. However, it is advisable to remove excess food, fecal material, and any solids that may have settled from the effluent, from the bottom of the test vessels daily by aspirating with a pipette.

PERSISTENCE OF EFFLUENT TOXICITY

The persistence of the toxicity of an effluent, expressed as its half-life, may be of interest in assessing the potential effects of the effluent on the receiving water (p. 122, USEPA, 1973). A rough approximation of the persistence of the toxicity can be obtained by measuring the 24-h LC50 of a portion of the sample immediately (within 36 h) following collection, and repeating the test on the remaining portion of the sample 96 h after the first test. The portion of the effluent set aside for the second test should be held in an uncovered glass container at room temperature, in direct sunlight if possible, and must be aerated.

If the results of the second test indicate that the toxicity of the effluent has decreased to 50% or less of its previous value during the storage period (i.e., if the LC50 increased from 10% to 20% or more), the toxicity can be considered to be nonpersistent (having a half-life of less than 96 h). If the toxicity has not decreased to 50% of its previous value, the toxicity of the effluent can be considered to be persistent. The method is limited to use with effluents having an initial LC50 of less than 50%.

SUMMARY OF TEST CONDITIONS FOR COMMONLY USED TEST ORGANISMS

Summaries of the test conditions for the daphnids, Daphnia pulex and D. magna, the mysid, Mysidopsis bahia, fathead minnows, Pimephales promelas, and the silverside, Menidia, are provided in Tables 5 - 8.

TABLE 5. RECOMMENDED TEST CONDITIONS FOR DAPHNIDS
(DAPHNIA PULEX^a AND D. MAGNA)

1. Temperature (°C):	20 ± 2°C
2. Light quality:	Ambient laboratory illumination
3. Light intensity:	50-100 footcandles (ft c) ^b (ambient laboratory levels)
4. Photoperiod:	8-16 h light/24 h
5. Size of test vessel:	100 mL beaker
6. Volume of test solution:	50 mL
7. Age of test animals:	1-24 h (neonates)
8. No. animals per test vessel:	10
9. No. of replicate test vessels per concentration:	2
10. Total no. organisms per concentration:	20
11. Feeding regime:	Feeding not required during first 48 h. For longer tests, feed every other day beginning on the third day (Appendix A).
12. Aeration:	None, unless DO concentration falls below 40% of saturation, at which time start gentle, single-bubble, aeration.
13. Dilution water:	Receiving water or other surface water, ground water, or synthetic water: hard water for <u>Daphnia magna</u> ; moderately hard or soft water for <u>D. pulex</u>
14. Test duration:	Screening test - 24 h (Static Tests) Definitive test - 48 h (Static Tests)
15. Effect measured:	Mortality - no movement of body or appendages on gentle prodding (LC50)

^a Use of D. pulex is preferred.

^b ft c = foot candles.

TABLE 6. RECOMMENDED TEST CONDITIONS FOR MYSIDS
(MYSIDOPSIS BAHIA)

1. Temperature (°C):	20 ± 2°C
2. Light quality:	Ambient laboratory illumination
3. Light intensity:	50-100 ft c (ambient laboratory levels)
4. Photoperiod:	8-16 h light/24 h
5. Size of test vessel:	250 mL
6. Volume of test solution:	200 mL
7. Age of test animals:	1-5 days
8. No. animals per test vessel (200 mL):	10
9. No. of replicate test vessels per concentration:	2
10. Total no. organisms per concentration:	20
11. Feeding regime:	Two drops of concentrated brine shrimp nauplii suspension twice daily (approx. 100 nauplii/mysid). See Appendix A.
12. Aeration:	None, unless dissolved oxygen concentration falls below 40% of saturation, at which time gentle single-bubble aeration should be started.
13. Dilution water:	Natural seawater, or synthetic salt water adjusted to 20 ppt salinity.
14. Test duration:	Screening test - 24 h (Static tests) Definitive test - 48 h (Static tests) - 48 to 96 h (Flow-thru)
15. Effect measured:	Mortality - no movement of body or appendages on gentle prodding (LC50)

TABLE 7. RECOMMENDED TEST CONDITIONS FOR THE FATHEAD MINNOW
(PIMEPHALES PROMELAS)

1. Temperature (°C):	20 ± 2°C
2. Light quality:	Ambient laboratory illumination
3. Light intensity:	50-100 ft c (ambient laboratory levels)
4. Photoperiod:	8-16 h light/24 h
5. Size of test vessels:	1 L
6. volume of test solution:	0.75 L
7. Age of fish:	1-90 days
8. No. of fish/0.75 L:	10 (Not to exceed loading limits)
9. No. of replicate test vessels/conc.:	2
10. Total no. organisms per concentration:	20
11. Feeding regime:	Feeding not required first 96 h. For longer tests, see Appendix A for feeding instructions.
12. Aeration:	None, unless DO concentration falls below 40% of saturation, at which time gentle single-bubble aeration should be started.
13. Dilution water:	Receiving water, other surface water, ground water, or soft synthetic water.
14. Test duration:	Screening test - 24 h (Static tests) Definitive test - 48 h (Static tests) - 48-96 h (Flow-thru)
15. Effect measured:	Mortality - no movement (LC50)

TABLE 8. RECOMMENDED TEST CONDITIONS FOR SILVERSIDES
(MENIDIA SPP.)

1. Temperature (°C):	20 + 2°C (Northern latitudes) 25 ± 2°C (Southern latitudes)
2. Light quality:	Ambient laboratory illumination
3. Light intensity:	50-100 ft c (ambient laboratory levels)
4. Photoperiod:	8-14 h light/24 h
5. Size of test vessel:	1 L
6. Volume of test solution:	0.75 L
7. Age of test animals:	1 to 90 days
8. No. of test fish/0.75 L:	10 (Not to exceed loading limits)
9. No. of replicate test vessels/conc.:	2
10. Total no. organisms per concentration:	20
11. Feeding regime:	Feeding not required first 96 h. For longer tests, see Appendix A for feeding instructions.
12. Aeration:	None, unless DO concentration falls below 40% of saturation, at which time gentle single-bubble aeration should be started.
13. Dilution water:	Natural seawater or reconstituted saltwater adjusted to 25-30 ppt salinity.
14. Test duration:	Screening test - 24 h (Static tests) Definitive test - 48 h (Static tests) - 48-96 h (Flow-thru)
15. Effect measured:	Mortality - No movement (LC50).

SECTION 9

EFFLUENT FRACTIONATION PROCEDURE

(Prepared by Gerald Walsh)

Depending on the objectives of the study and the resources available, the source of toxicity in an effluent can be characterized by subjecting it to a series of fractionations using ion exchange resins (Fig. 4). In this approach, liquid effluent that exhibits toxicity is filtered to remove the particulate matter, the filtrate is passed through an XAD-4 resin to separate the organic and inorganic fractions, and the toxicity tests are conducted with the two fractions. If either fraction exhibits toxicity, subfractionation may be carried out using ion exchange resins for the inorganics and extraction under basic and acidic conditions for the organics. Toxicity test may be conducted on the subfractions. Identification of possible toxic substances may be made by inductively coupled plasma (ICAP), high performance liquid chromatography (HPLC), and gas chromatography mass spectrometry (GCMS) analyses. Such analyses of all subfractions are informative because some organic molecules behave as ions and may be found occasionally in the "inorganic" fraction. For a detailed discussion of the fractionation and toxicity test procedure, see Walsh and Garnas (1983).

SECTION 10

TEST DATA

BIOLOGICAL DATA

Death is the "effect" used for determining toxicity to aquatic organisms in acute toxicity tests. The criteria usually employed in establishing death of fish are: (1) no movement, especially no gill movement; and (2) no reaction to gentle prodding.

Death is not as easily determined for some invertebrates, such as daphnids, and is defined as the lack of movement of the body or appendages on gentle prodding. However, the death of some invertebrates, such as mysids, is easily detected because of a change in pigmentation from transparent or translucent to opaque. General observations of appearance and behavior (see Table 9), such as erratic swimming, loss of reflex, discoloration, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, and cannibalism, should also be noted in the daily log.

The number of surviving organisms in each test container should be counted at one, two, four and eight hours after the beginning of a screening test, and daily (Figs. 5 and 6) in definitive tests. When recognizable, dead organisms should be removed during each observation period.

The mean lengths and weights of test fish are obtained by sacrificing 10 animals from the original pool of test organisms at the beginning of the test, or using the control organisms at the conclusion of the test. The species, source, and age of the test organisms should also be recorded.

CHEMICAL AND PHYSICAL DATA

In nonrenewal static tests, at a minimum, pH, and salinity or conductivity, and total residual chlorine are measured in the control and in the highest effluent concentration at the beginning of the test. The DO should be monitored closely (every 2 h) for the first 4 to 8 h as described above, and measured daily thereafter in all effluent concentrations in which there are surviving organisms. Temperature is measured at the beginning of the test, and daily thereafter. It is recommended that total alkalinity and total hardness be measured in the control and highest effluent concentration at the beginning of the test.

TABLE 9. DEFINITION OF FISH BEHAVIOR TERMS^a

TERM	DEFINITION
GENERAL BEHAVIOR -	Observable responses of the test fish, individually or in groups, to their environment.
a. Quiescent:	Marked by a state of inactivity or abnormally low activity; motionless or nearly so.
b. Hyperexcitable:	Reacting to stimuli with substantially greater intensity than control fish.
c. Irritated:	Exhibiting more or less continuous hyperactivity.
d. Surfacing:	Rising and remaining unusually long at the surface.
e. Sounding:	Diving suddenly straight to the bottom; remaining unusually long at the bottom.
f. Twitching:	Moving the body or parts of the body with sudden jerky movements.
g. Tetanous:	In a state of tetany; marked by intermittent tonic spasms of the voluntary muscles.
h. Flaccid:	Lacking tone, resilience or firmness; weak and enfeebled; flabby.
i. Normal:	Unaffected by or not exposed to a particular experimental treatment; conforming to the usual behavioral characteristics of the species.
SWIMMING -	Progressive self-propulsion in water by coordinated movement of tail, body, fins.
a. Ceased:	Broken off or tapered off to a stop.
b. Erratic:	Characterized by lack of consistency, regularity, or uniformity; fluctuating, uneven; eccentric.
c. Gyrating:	Revolving around a central point; moving spirally about an axis.
d. Skittering:	Skimming hurriedly along the surface with rapid body movements.
e. Inverted:	Turned upside down, or approximately so.
f. On side:	Turned 90° laterally, more or less, from the normal body orientation.
PIGMENTATION -	Color or skin due to deposition or distribution of pigment.
a. Light discolored:	Color appearance lighter than usual for the species.
b. Dark discolored:	Color appearance darker than usual for the species.
c. Varidiscolored:	Color appearance abnormally varied; mottled.
INTEGUMENT -	The skin.
a. Mucus shedding:	Observably losing mucous skin coating to an abnormal degree.
b. Mucus coagulation:	Showing observable clumping or clotting of the mucous skin coating, especially at the gills.
c. Hemorrhagic:	Visibly bleeding as from gills, eyes, anal opening.
RESPIRATION -	Physical action of pumping water into mouth and out through gills, so as to absorb oxygen.
a. Rapid:	Observably faster than normal to a significant degree.
b. Slow:	Observably slower than normal to a significant degree.
c. Irregular:	Failing to occur at regular or normal intervals.
d. Ceased:	Broken off or tapered off to a stop.
e. Gulping air:	Swimming at surface with mouth open and laboriously pumping surface water and air through gills.
f. Labored:	Performed with apparent abnormally great difficulty and effort.

^aFrom Brusick and Young, 1981.

1. EXPOSURE CHAMBER

2. FEEDING SCHEDULE

Total vessel capacity: _____ mL

Not fed: _____

Test solution volume: _____ "

Fed daily: _____

Water depth: constant - _____ cm

Fed irregularly (describe): _____

cyclic - _____ to _____ cm.

3. AERATION

4. SCREENED ANIMAL ENCLOSURES

None: _____

Not used: _____

Slow: _____ (mL/min)

Used: _____ (cm) Diameter

Moderate: _____ "

Vigorous: _____ "

Beginning _____ (hour)

5. Condition of surviving organisms at end of test: _____

6. Comments: _____

Figure 6. Check list on back of effluent toxicity data sheet.

In flow-through and static renewal tests, at a minimum, pH, salinity or conductivity, total alkalinity, total hardness, and total residual chlorine are measured daily in the highest effluent concentration. DO and temperature are measured at the beginning of the test and daily thereafter in the control and all all effluent concentrations containing surviving test organisms.

The measurement of specific conductance is recommended because it is a very useful parameter in detecting transient fluctuations in the chemical characteristics of effluents, and will indicate errors in test dilutions.

Water samples collected for chemical analysis should be taken midway between the top, bottom, and sides of the test containers, and should not include any surface scum or material stirred up from the bottom or sides.

Methods used for chemical analysis should be those specified for Section 304(h) of the CWA (USEPA 1979b, 1982). For salinity measurements, a refractometer may be used if calibrated with a sample of known salinity. Temperature should be recorded continuously in all tests in at least one chamber.

SECTION 11

TOXICITY DATA ANALYSIS

INTRODUCTION

Data from acute effluent toxicity tests are used to estimate the median lethal effluent concentration, or LC50, which is the concentration that is lethal to 50% of the organisms within the test period. Only rarely will a given effluent concentration result in exactly a 50% mortality of the test organisms. Therefore, some mathematical means, such as regression analysis, is generally needed to obtain an estimate of the LC50 by interpolation between two concentrations. Also, because of the normal variation in sensitivity of individuals within a group of test organisms, there is a degree of uncertainty regarding the "true" or "exact" value of the LC50. This uncertainty is expressed as a confidence interval, or range of values within which the "true" LC50 could occur. The confidence interval is defined in terms of the level of probability (usually 0.95) that the "true" LC50 is included.

When plotted on linear graph paper, mortality data from a toxicity test which results in partial mortalities at several concentrations of the toxicant typically form an "S"-shaped, or sigmoid, curve (Fig. 7). The major portion of the curve will approximate a straight line if the concentrations are converted (transformed) to logarithms, or if the data are plotted on semi-log paper. Linearity in the relationship between concentration and mortality is most closely achieved in the vicinity of

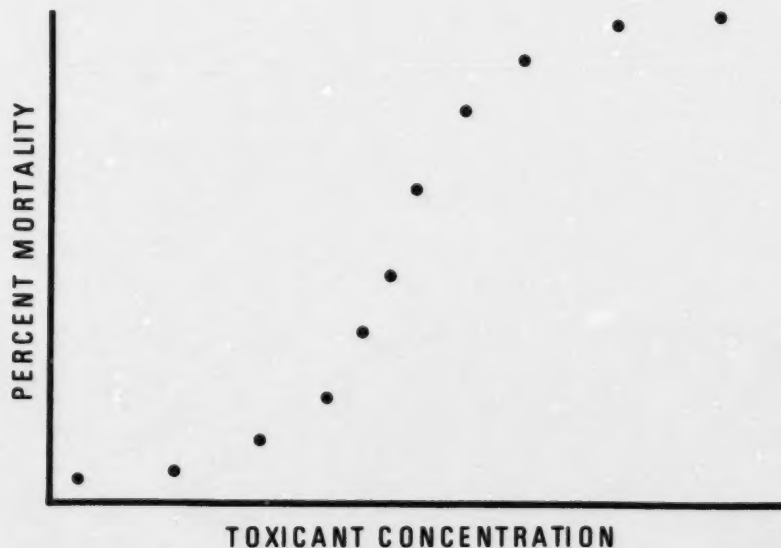


Figure 7. Linear plot of toxicity data.

the LC50, in the central region of the curve. The LC50, therefore, is the statistic that can be estimated with the greatest certainty. Transformations of the data permit more exact estimates of the LC50 and facilitate the calculation of confidence intervals. The similarity in the shape of toxicity data curves after transformation of the data to Probits, Logits, and angles is shown in Fig. 8. Commonly used methods of estimating the LC50 and its confidence interval include the graphical, Litchfield-Wilcoxon, binomial, Probit, Logit, moving average, moving average-angle, and Spearman-Kärber methods (Finney, 1971; Stephan, 1977; Peltier, 1978a,b). Fortunately, the availability of inexpensive but powerful scientific pocket calculators and computers has eliminated most of the tedium previously associated with the use of some of these methods.

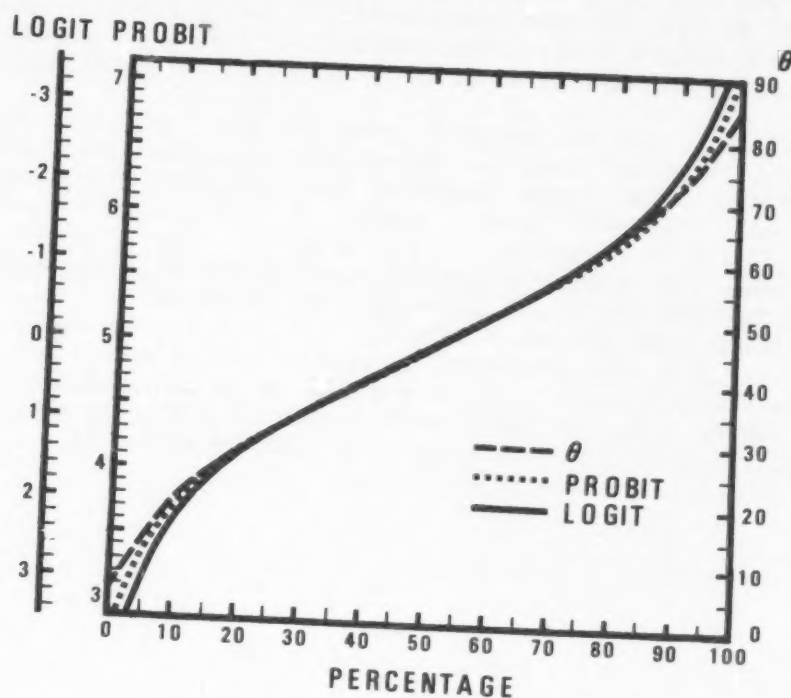


Figure 8. Comparison of angular, Logit, and Probit transformations of toxicity data. (From Knudsen and Curtis, 1947.)

ALL-OR-NOTHING NATURE OF MORTALITY DATA FROM EFFLUENT TOXICITY TESTS

Unlike tests with pure compounds, which usually result in a regular progression in percent mortality with increasing toxicant concentration, toxicity tests with effluents commonly yield all-or-nothing results, with no concentrations showing partial mortalities. In these cases, exposures to one or more of the higher concentrations of effluent (such as 100% and 50% effluent) result in 100% mortality of the test organisms, whereas exposures at lower concentrations of effluent (such as 25%, 12.5%, and 6.25%) all result in 100% survival. These results obviate the use of some

methods of calculating the LC50, such as the Probit, Litchfield-Wilcoxon, and Spearman-Kärber analyses. Of 60 effluent toxicity tests conducted during FY-83 by the Environmental Services Division, USEPA Region 4, Athens, Georgia, in which test organism lethality occurred, only four (7%) were amenable to Probit analysis.

VARIABILITY IN TOXICITY TEST RESULTS

As was mentioned earlier, the toxicity test results will depend upon the species used, the strain or source of the test organisms, the condition or health of the organisms, and test conditions such as temperature, DO, food, water quality, etc. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (numbers of fish) and the standard error of the test, and considered 20 fish/concentration as optimum.

Test precision can be estimated by using the same strain of organisms under the same test conditions, and employing a known toxicant, such as a reference toxicant. The single laboratory and multi-laboratory precision of acute toxicity tests with several common test species and reference toxicants is listed in Tables 10, 11, and 12 below. The values for single laboratory precision (expressed as coefficient of variation) from 92 reference toxicant tests with three species, as listed in Table 10, ranged from 10% to 86%, and had a weighted mean of 38%. The values for multi-laboratory precision (expressed as coefficient of variation) from 153 reference toxicant tests with six species, as listed in Table 11, ranged from 22% to 167%, and had a weighted mean of 50%. The multi-laboratory precision of toxicity tests with standard effluents is also 30% to 50% (see Table 12 and Buikema, 1983).

TABLE 10. SINGLE LABORATORY PRECISION OF STATIC ACUTE TOXICITY TESTS
WITH AQUATIC ORGANISMS, USING REFERENCE TOXICANTS^{a,b}

TEST ORGANISM	REFERENCE TOXICANT					
	SDS		NAPCP		CD	
	N	CV(%)	N	CV(%)	N	CV(%)
<i>Pimephales promelas</i> (Fathead Minnow) (96 h)	9	22	12	40	9	86
<i>Daphnia pulex</i> (48 h)	10	43	14	36	9	21
<i>Daphnia magna</i> (48 h)	8	29	13	10	8	72

- a. SDS = Sodium dodecyl (lauryl) sulfate
 NAPCP = Sodium pentachlorophenate
 CD = Cadmium
 N = Number of tests
 CV(%) = Coefficient of variation = (standard deviation x 100)/mean.

- b. Data provided by Philip Lewis and James Dryer, Aquatic Biology
 Section, EMSL-Cincinnati, and taken in part from Lewis and
 Weber, 1985.

TABLE 11. MULTI-LABORATORY PRECISION OF ACUTE TOXICITY TESTS
WITH AQUATIC ORGANISMS, USING REFERENCE TOXICANTS^a

TEST ORGANISM	REFERENCE TOXICANT			
	SILVER NITRATE		ENDOSULFAN	
	N	CV(%) ^b	N	CV(%)
1. <u>Pimephales promelas</u>				
96-h static test	10	52	12	38
96-h flow-through test	10	40	12	46
2. <u>Salmo gairdneri</u>				
96-h static test	10	64	12	50
96-h flow-through test	10	32	12	43
3. <u>Daphnia magna</u>				
48-h static	8	71	12	51
4. <u>Acartia tonsa</u>				
96-h static test	5	42	6	82
5. <u>Mysidopsis bahia</u>				
96-h static test	6	27	5	62
96-h flow-through test (Nom)	6	22	6	58
96-h flow-through test (Meas)	6	58	5	167
6. <u>Cyprinodon variegatus</u>				
96-h static test (Nom)	4	35	6	37
96-h flow-through test (Nom)	5	50	6	46
96-h flow-through test (Meas)	5	46	6	38

a. Data for Pimephales promelas (fathead minnow), Salmo gairdneri (rainbow trout), and Daphnia magna were taken from Broderius, 1983. Data for Acartia tonsa, Mysidopsis bahia, Cyprinodon variegatus (sheepshead minnow) were taken from Schimmel, 1981. Six laboratories participated in each study.

In the studies with the freshwater organisms, the water hardness for five of the six laboratories ranged between 36 and 75 mg/L. However, the water hardness for the sixth laboratory was 255 mg/L, resulting in

TABLE 11. Footnotes (continued)

LC50 values for silver nitrate more than an order of magnitude larger than for the other five. These values were rejected in calculating the CV%.

N, the total number of LC50 values used in calculating the CV, varied with organism and toxicant because some data were rejected due to water hardness, and/or because some of the LC50s were not calculable.

In studies with the marine organisms, only one LC50 (presumably the combined LC50 from duplicate tests) was reported for each toxicity test, and N = 6 in each case. LC50s for the 96-h flow-through tests with Mysidopsis bahia and Cyprinodon variegatus were calculated two different ways -- (1) on the basis of the nominal toxicant concentrations (Nom), and (2) on the basis of measured (Meas) toxicant concentrations.

b. $CV(\%) = \text{Coefficient of variation} = (\text{standard deviation} \times 100) / \text{mean}.$

TABLE 12. MULTI-LABORATORY PRECISION OF 48-H ACUTE TOXICITY TESTS
WITH DAPHNIA MAGNA, USING A STANDARD EFFLUENT^{a,b}

Laboratory	LC50s From Replicate Tests	Mean LC50	SD ^c	CV(%) ^d
<u>Industry</u>				
1	4.2 4.9	4.6		
2	6.8 6.1 6.1	6.3	0.40	6.4
3	3.5 7.1	5.3		
<u>Government</u>				
1	4.4 4.4 4.1	4.3	0.17	4.0
2	4.5 4.5	4.5		
3	4.2	--		
<u>Commercial</u>				
1	4.9 4.7	4.8		
2	3.7 5.6	4.6		
3	9.0 9.1 8.6	8.9	0.26	3.0
	Mean 5.5 SD 1.8 CV(%) 31.8			

a. From Table 2, p. 191, Grothe and Kimerle, 1985.

b. LC50 expressed in percent effluent.

c. SD = Standard deviation; determined only for three or more replicates.

d. CV(%) = (Standard deviation X 100)/Mean

MANUAL METHODS FOR ESTIMATING THE LC50

1. Graphical Method

The first step in the evaluation of the toxicity test data should consist of the preparation of a plot of the data using 2-cycle semi-log graph paper (Fig. 9). This technique is commonly referred to as the Log-Concentration-Versus-Percent-Mortality method. The logarithmic axis (y axis) is used for percent effluent concentration, and the linear (x axis) is used for percent mortality. The graph is useful in providing: (1) a rapid overview of the distribution of the data that will aid in determining if there is a regular progression of mortality with increasing concentration, especially in the central region of the curve; and (2) a reasonably-accurate estimate of the LC50. However, the method does not provide a confidence interval around the estimate of the LC50.

General Procedure:

- Plot the percent effluent volumes and the corresponding percent mortality on semi-logarithmic graph paper as described above.
- Locate the two points on the graph which are separated by the 50% mortality line, and connect them with a straight line (see Fig. 9). However, if one of the points is obviously irregular, the mortality value for the next lower or higher percent effluent volume is used.
- On the scale for percent effluent volume, read the value of the point where the diagonal line and the 50% mortality line intersect. This value is the LC50 for the test, expressed as a percent effluent volume.

Example:

Step 1. The percent effluent volumes and the corresponding percent mortality values listed below are plotted in Fig. 9.

Effluent Concentration (%)	Percent Mortality
100.0	100
50.0	100
25.0	0
12.5	0
6.25	0

Step 2. The two adjacent points which are separated by the 50% mortality line (50% effluent and 25% effluent) are located and connected with a diagonal straight line.

Step 3. An LC50 of 35% effluent was derived for the test by determining the effluent concentration at the point where the diagonal line drawn between 100% and 0% mortality intersected the 50% mortality line.

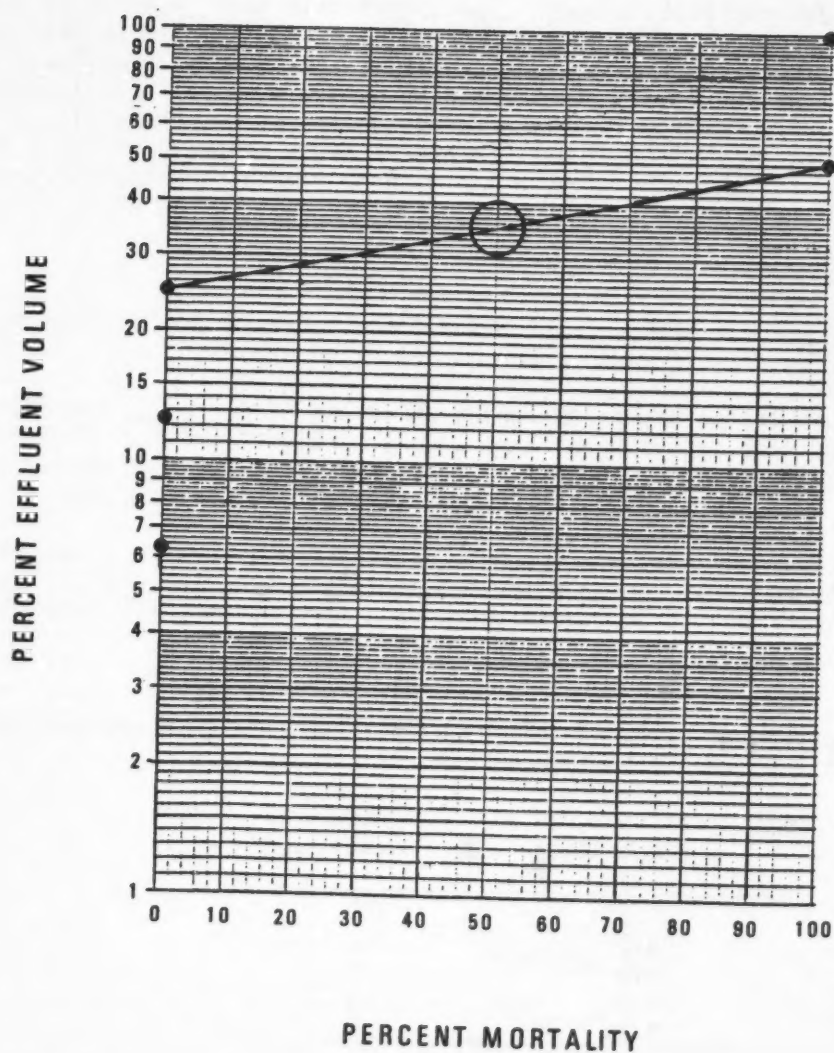


Figure 9. Plotted data and fitted line for log-concentration-versus percent-mortality method of determining LC50.

2. Moving Average-angle Method

The moving average-angle method is recommended for use in calculating the LC50 and its confidence interval for effluent toxicity data for two important reasons:

- a. It permits the calculation of the 95% confidence interval for the LC50 when no partial mortalities occur in a test, which is a common occurrence in effluent bioassays, and
- b. The method lends itself readily to the use of inexpensive, scientific pocket calculators or pocket computers, that have log, antilog, and inverse sine or inverse tangent functions.

The use of the method, however, is limited to those data sets in which there are two effluent concentrations above the LC50.

Example of Calculation of the LC50 and 95% Confidence Interval:

An example of an LC50 calculation with the moving average-angle method is provided below, based on the data listed on p. 57 and shown in Fig. 9:

Step 1. Prepare a table using the data from an effluent toxicity test and carry out the calculations as illustrated below.

TABLE 13. SAMPLE DATA FOR MOVING AVERAGE-ANGLE METHOD

Effluent Concentration (%)	Transformed Effluent Concentration (Log ₁₀)	Response Proportion (RP)	Transformed RP (Degrees)	Moving Average-Angle (Degrees)
(a)	(b)	(c)	(d)	(e)
100.0	2.0000	1.0	83.5807	
50.0	1.6990	1.0	83.5807	57.8602
25.0	1.3979	0.0	6.4193	32.1398
12.5	1.0969	0.0	6.4193	*
6.25	0.7959	0.0	6.4193	*

*No calculation required (see Step 5, below).

Step 2. List the effluent concentrations (as percent effluent) and log₁₀ of the concentration of each treatment level (see columns "a" and "b" in the table above).

Step 3. Calculate the response proportion (RP) at each effluent concentration (see column "c" in the table above), where:

$$RP = (\text{number of dead or "affected" organisms})/(\text{initial number}).$$

Step 4. Transform each RP to an angle, using $\text{Arcsin}(\text{Sin}^{-1})$, as follows (see column "d" in the table above):

a. For $\text{RP} = 0.01$ to 0.99 : (Eq. 1)

$$\text{Angle} = \text{Arcsin}(\text{RP})^{0.5}$$

b. For $\text{RP} = 0$ (no mortality) or 1 (100% mortality), the following adjustments are made:

(1) For $\text{RP} = 0$ (no mortality): (Eq. 2)

$$\text{Angle} = \text{Arcsin}(1/4N)^{0.5}$$

Where: N = Number of animals/treatment

Example: If 20 animals are used,

$$\begin{aligned}\text{Angle} &= \text{Arcsin}(1/80)^{0.5} \\ &= \text{Arcsin } 0.1118 \\ &= 6.4193 \text{ degrees}\end{aligned}$$

(2) For $\text{RP} = 1.0$ (100% mortality):

$$\text{Angle} = 90.0 - (\text{angle for } \text{RP} = 0) \quad (\text{Eq. 3})$$

Example: Using above value

$$\begin{aligned}\text{Angle} &= 90.0 - 6.4193 \\ &= 83.5807 \text{ degrees}\end{aligned}$$

Step 5. Calculate the moving average-angles as follows, until two average angles are obtained that bracket 45° (see column "e" in the table above):

a. Sum the largest three angles and divide by 3:

$$(83.5807 + 83.5807 + 6.4193)/3 = 57.8602$$

b. Sum the next largest three angles and divide by 3:

$$(83.5807 + 6.4193 + 6.4193)/3 = 32.1398$$

c. These two angles bracket 45° . No further averaging is required.

d. Proceed to Step 6 (LC50 calculation).

Step 6. Calculate the LC50:

a. Calculate A:

$$A = \frac{45.0 - \text{LMAA}}{\text{UMAA} - \text{LMAA}} = \frac{45.0 - 32.1398}{57.8602 - 32.1398} = \frac{12.8602}{25.7204} = 0.5000 \quad (\text{Eq. 4})$$

Where: 45° = Position of the LC50 (percent effluent)

LMAA = (Lower Moving Average-Angle) The angle less than but closest to 45° (32.1398 degrees in the above example).

UMAA = (Upper Moving Average-Angle) The angle greater than, but closest to 45° (57.8602 in the above example).

b. Using A above, calculate the LC50:

$$\begin{aligned} (1) \log_{10} \text{LC50} &= \text{LLC} + (\text{LUC} - \text{LLC})(A) & (\text{Eq. 5}) \\ &= \text{LOG}(25) + ((\text{LOG}(50) - \text{LOG}(25))(A)) \\ &= 1.3979 + (1.6990 - 1.3979)(0.5000) \\ &= 1.5485 \end{aligned}$$

Where: LLC = Log Lower Concentration, or $\log_{10}$ of the effluent concentration just below, but closest to, $\text{RP} = 0.5$ (1.3979 in above example)

LUC = Log Upper Concentration, or $\log_{10}$ of the effluent concentration just above but closest to, $\text{RP} = 0.5$ (1.6990 in above example)

$$(2) \text{LC50} = 10^{1.5485} = 35.35\% \text{ Effluent}$$

Step 7. Calculate the 95% confidence limits for the LC50:

a. Calculate G:

$$G^a = \frac{1641.4(1.96)^2}{(N)(K^2)(\text{UMAA} - \text{LMAA})^2} = \frac{6305.6}{(20)(9)(25.7209)^2} = 0.05295 \quad (\text{Eq. 6})$$

Where: K = Number of angles averaged (3)
N = Number of organisms per treatment (20)
 $2(90/n)^2 = 1641.4$

^aFor further reference to "G," see Appendix E.

b. Calculate AG:

$$AG = \frac{A - G/2}{1 - G} = \frac{0.5000 - (0.05295/2)}{1 - 0.05295} = 0.5000 \quad (\text{Eq. 7})$$

c. Calculate AU and AL:

$$\begin{aligned} (1) \text{ AU} &= AG + (G^{0.5}/(1 - G))((A - 0.5)^2 + 5/4(1 - G))^{0.5} \\ &= 0.5000 + (0.2301/0.9470)((.5000-0.5)^2 + 5/4(.9470))^{0.5} \\ &= 0.5000 + (0.2430)(1.088) \\ &= 0.5000 + 0.2644 = 0.7644 \end{aligned}$$

$$\begin{aligned} (2) \text{ AL} &= AG - (G^{0.5}/(1 - G))((A - 0.5)^2 + 5/4(1 - G))^{0.5} \\ &= 0.5000 - 0.2644 = 0.2356 \end{aligned}$$

d. Calculate the 95% confidence limits of LC50:

(1) Upper confidence limit (UCL): (Eq. 8)

$$\begin{aligned} (a) \text{ Log}_{10} \text{ UCL} &= LLC + (LUC - LLC)(AU) \\ &= 1.3979 + (1.6990 - 1.3979)(.7644) \\ &= 1.3979 + 0.2302 = 1.6281 \end{aligned}$$

$$(b) \text{ UCL} = 10^{1.6281} = 42.47\%$$

(2) Lower confidence limit (LCL):

$$\begin{aligned} (a) \text{ Log}_{10} \text{ LCL} &= LLC + (LUC - LLC)(AL) \\ &= 1.3979 + (1.6990 - 1.3979)(0.2356) \\ &= 1.4688 \end{aligned} \quad (\text{Eq. 9})$$

$$(b) \text{ LCL} = 10^{1.4688} = 29.43\% \text{ effluent}$$

Step 8. Results:

a. LC50 = 35.35% effluent

b. The 95% confidence interval = 29.4% - 42.5% effluent

3. Litchfield - Wilcoxon Abbreviated Method¹

This method cannot be used unless partial mortalities occur in the test.

General Procedure:

- a. Tabulate the data showing the percent-effluent volumes used, the total number of organisms exposed to each percent-effluent volume, the number of affected organisms, and the observed percent-effluent organisms (see Table 14). Do not list more than two consecutive 100% affects at the higher percent effluent volumes or more than two consecutive 0% affects at the lower percent-effluent volumes.
- b. Plot the percent-affected organisms against the percent-effluent volume on two-cycle, logarithmic probability paper (Fig. 10), except for 0% or 100% affect values. With a straight edge, fit a temporary line through the points, particularly those in the region of 40% to 60% affects.
- c. Using the line drawn through the points, read and list an "expected" percent effect for each percent-effluent volume tested. Disregard the "expected" percent value for any of the percent volumes less than 0.01 or greater than 99.99. Using the expected-percent-affect, calculate from Table 15 a "corrected" value for each 0% or 100% affect obtained in the test. (Since the expected values in the table are whole numbers, it will be necessary to obtain intermediate values by interpolation.) Plot these values on the logarithmic probability paper (Fig. 10) used in Step 2 and inspect the fit on the line to the completely plotted data. If after plotting the corrected expected values for 0% and 100% affected, the fit is obviously unsatisfactory, redraw the line and obtain a new set of expected values.
- d. List the difference between each observed (or corrected) value and the corresponding expected value. Using each difference and the corresponding expected value, read and list the contributions to Chi-square (χ^2) from Fig. 11. (A straight edge connecting a value on the Expected-Percent-Affected scale with a value on the Observed-Minus-Expected scale, will indicate at the point of intersection of the χ^2 scale, the contribution to χ^2). Sum the contributions to χ^2 and multiply the total by the average number of organisms per effluent volume, i.e., the number of organisms used in K concentrations divided by K, where K is the number of percent-affected organism values plotted. The product is the "calculated" χ^2 of the line. The degrees of freedom (N) are 2 less than the number of points plotted, i.e., $N = K - 2$. If the calculated χ^2 is less than the χ^2 given in Table 16 for N degrees of freedom, the data are non-heterogeneous and the line is a good fit. However, if the calculated χ^2 is greater than the χ^2 given in Table 16 for N degrees of freedom, the data are heterogeneous and the line is not a good fit. In the even a line cannot be fitted (the calculated χ^2 is greater than the tabular χ^2), the data can not be used to calculate an LC50 or EC50. Litchfield and

¹Taken from Peltier, 1978a,b.

Wilcoxon provided an alternate method for calculating the 95% confidence limits under these circumstances. However, the toxicity test should be repeated.

e. Determine the 95% confidence limits of the LC50.

(1) Read from the fitted line (Fig. 10), the percent effluent volumes for the corresponding 16%, 50%, and 84% affects (LC16, LC50, and LC84).

(2) Calculate the slope function, S, as:

$$S = \frac{LC84/LC50 + LC50/LC16}{2}$$

(3) From the tabulation of the data determine N', which is defined as the total number of test organisms used within the percent-affected-organism interval of 16% and 84%. Calculate the exponent $(2.77/\sqrt{N'})$ for the slope function and the factor, f_{LC50} , used to establish the confidence limits for the LC50 (or EC50).

$$f_{LC50} = S^{(2.77/\sqrt{N'})}$$

The f_{LC50} can be obtained directly from the nomogram in Fig. 12 by laying a straight-edge across the appropriate base and exponent values and reading the resultant "f" value.

(4) Calculate the 95% confidence limits of the LC50 as follows:

- (a) Upper limit = $LC50 \times f_{LC50}$
- (b) Lower limit = $LC50/f_{LC50}$

Example:

Steps 1-4. The data are tabulated, as in Table 14 (Step 1), plotted (Step 2, Fig. 10), and the expected values are read from the graph (Step 3). χ^2 is calculated (Step 4) as follows:

a. Mean number of organisms used in 'K' ($K = 6$) concentrations = $120/6 = 20$

(Note that the data for the 10% effluent volume were aberrant and were not used. Therefore, $K = 6$, and the total number of organisms = 120)

b. Calculate $\chi^2 = 20 \times 0.057 = 1.14$

c. Degrees of Freedom (N) = $K - 2 = 6 - 2 = 4$

d. From Table 16, the χ^2 for 4 degrees of freedom = 9.49

e. The calculated χ^2 is less than the tabular χ^2 . Therefore, it is assumed the line is a good fit, and the data are non-heterogeneous.

TABLE 14. SAMPLE DATA FOR LITCHFIELD-WILCOXON ABBREVIATED METHOD

Percent Effluent Volume	STEP ONE			STEP THREE	STEP FOUR	
	Number of Organisms	Number of Affected Organisms	Observed % Affected Organisms	Expected % (Fig. 10)	Observed Minus Expected	Chi ²
3.2	20	0	0 (0.2) ^a	0.6	0.4	0.003
5.6	20	1	5	3.5	1.5	0.006
10.0	20	11	55	(14.5) ^b	Aberrant	Value
18.0	20	7	35	38.0	3.0	0.004
32.0	20	12	60	67.0	7.0	0.024
56.0	20	18	90	87.5	2.5	0.006
100.0	20	20	100 (99.0) ^a	97.0	2.0	0.014
TOTAL						0.057

a. Step 3 "Corrected" affected values from Table 15.

b. Percent-affected organisms at the 10% effluent volume is obviously an aberrant value and should be omitted when fitting the line in Step 2.

Step 5. Determine the LC50 and 95% confidence limits.

a. From the fitted line in Fig. 10, determine the (percent) effluent concentrations corresponding to the 16%, 50%, and 84% affected organism values:

- (1) LC84 effluent concentration = 50.0%
- (2) LC50 " " = 23.0%
- (3) LC16 " " = 10.5%

b. Calculate the slope function, 'S', as:

$$S = \frac{LC84/LC50 + LC50/LC16}{2} = \frac{50.0/23.0 + 23.0/10.5}{2}$$

$$= \frac{2.17 + 2.19}{2} = \frac{4.36}{2} = 2.18$$

c. Determine N': N' = 40 (From Fig. 10).

d. Calculate the exponent (2.77/ N') and factor, f_{LC50}:

$$\begin{aligned} f_{LC50} &= S^{(2.77/ N')} = 2.18^{(2.77/ 40)} = 2.18^{(0.06925)} \\ &= 2.18^{(0.438)} = 1.41 \end{aligned}$$

e. Calculate the 95% confidence limits of the LC50:

- (1) Upper limit = LC50 × f_{LC50} = 23.0 × 1.41 = 32.4%
- (2) Lower limit = LC50/f_{LC50} = 23.0/1.41 = 16.3%

TABLE 15. CORRECTED VALUES FOR 0% OR 100% EFFECT

Expected Value	Corrected Value									
	0	1	2	3	4	5	6	7	8	9
0	--	0.3	0.7	1.0	1.3	1.6	2.0	2.3	2.6	2.9
10	3.2	3.5	3.8	4.1	4.4	4.7	4.9	5.2	5.5	5.7
20	6.0	6.2	6.5	6.7	7.0	7.2	7.4	7.0	7.8	8.1
30	8.3	8.4	8.6	8.8	9.0	9.2	9.3	9.4	9.6	9.8
40	9.9	10.0	10.1	10.2	10.3	10.4	10.4	10.4	10.4	10.5
50	--	89.5	89.6	89.6	89.6	89.7	89.7	89.8	89.9	90.0
60	90.1	90.2	90.4	90.5	90.7	90.8	91.0	91.2	91.4	91.6
70	91.7	91.9	92.2	92.4	92.6	92.8	93.0	93.3	93.5	93.8
80	94.0	94.3	94.5	94.8	95.1	95.3	95.6	95.9	96.2	96.5
90	96.8	97.1	97.4	97.7	98.0	98.4	98.7	99.0	99.3	99.7

TABLE 16. VALUES OF χ^2 ($P = 0.05$)

Degrees of Freedom (N)	χ^2
1	3.84
2	5.99
3	7.82
4	9.49
5	11.1
6	12.6
7	14.1
8	15.5
9	16.9
10	18.8

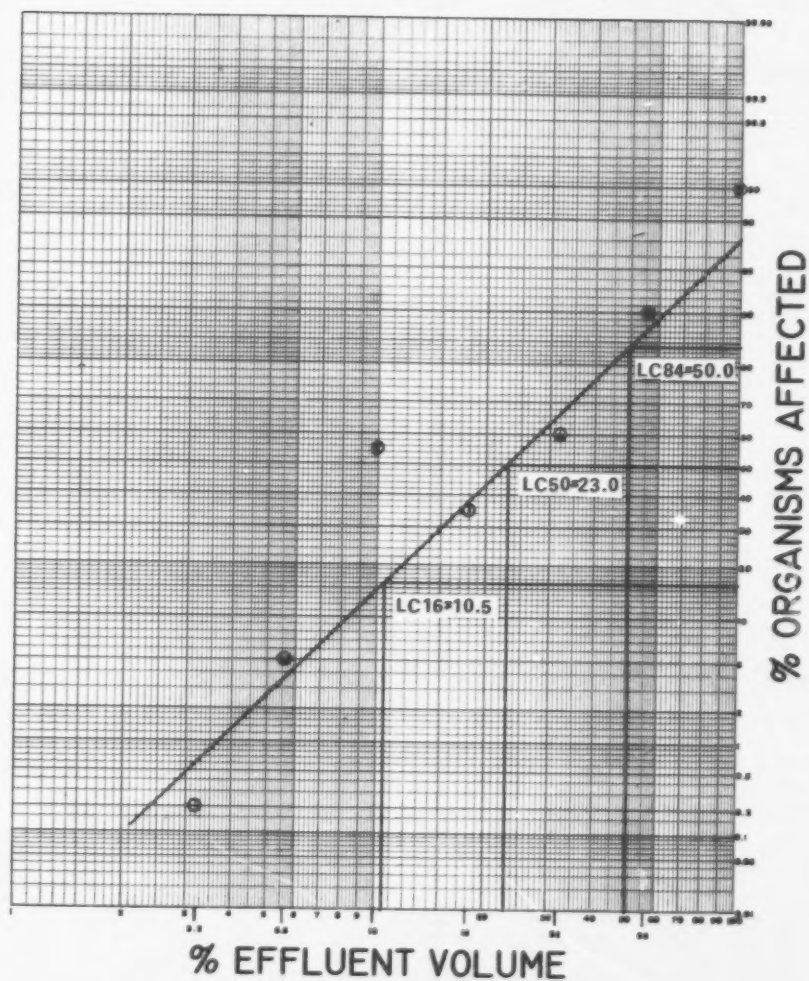


Figure 10. Line fitted to data, and LC16, LC50, and LC84, as read from the line (Steps 2, 3, and 4).

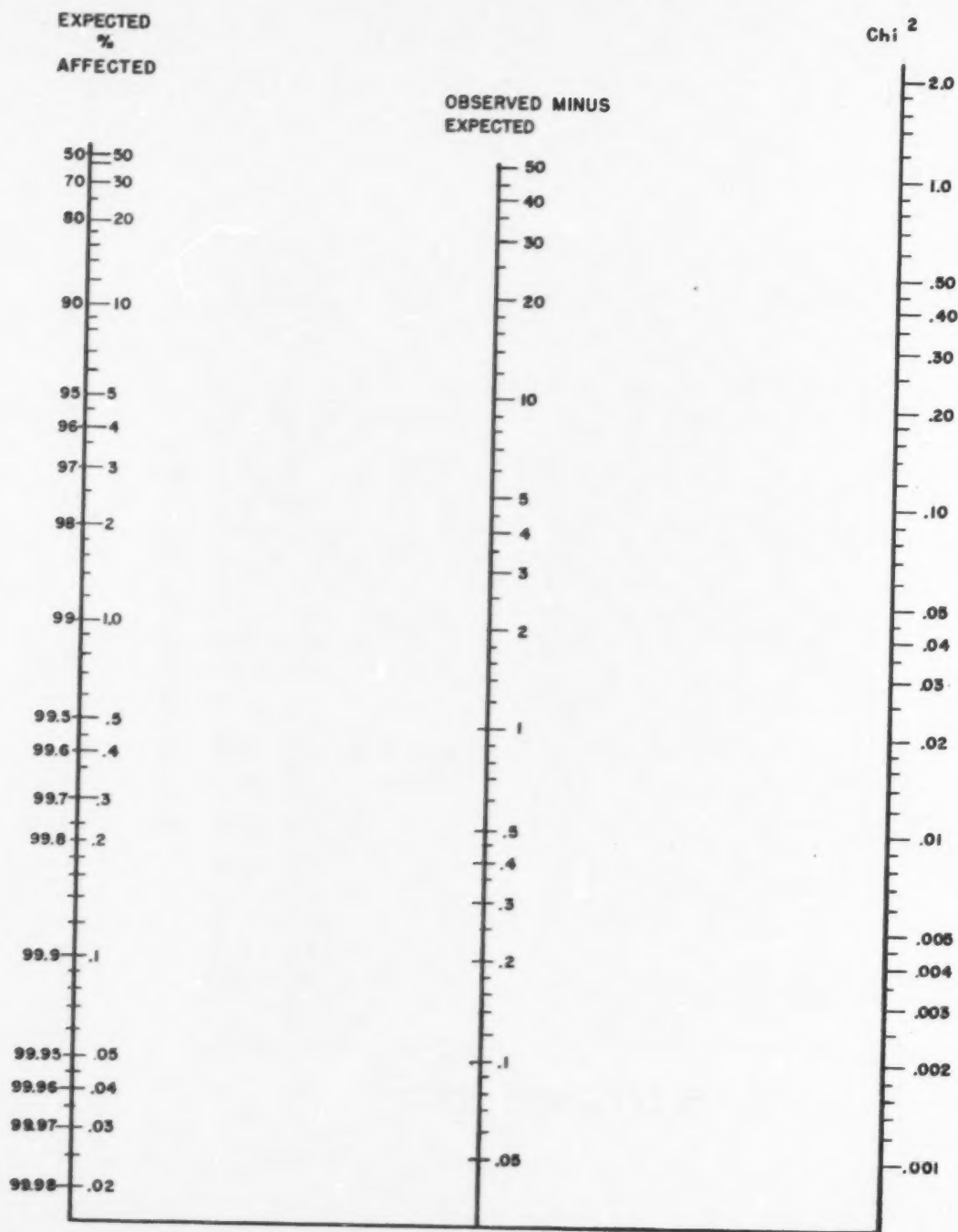


Figure 11. Nomograph for obtaining Chi² from expected-percent-affected and observed-minus-expected values (Step 4).

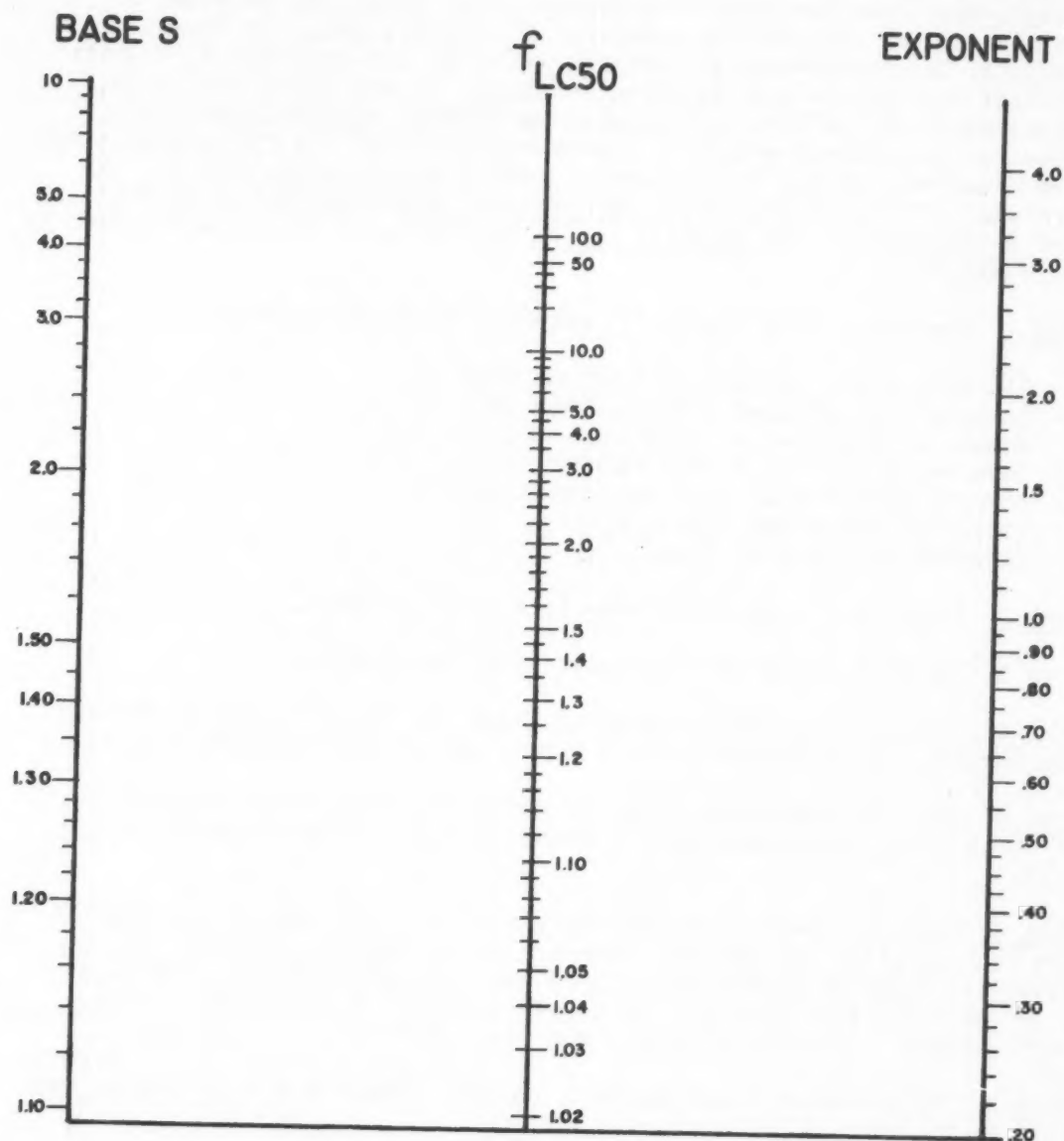


Figure 12. Nomograph for raising Base S to a fractional exponent.

4. Probit Method

Probit analysis consists of a group of statistical methods used to analyze data from concentration-response experiments, and provides an estimate of the LC50 and the precision of this estimate. In Probit analysis, the percentages of affected organisms are converted to Probits (Probability Units), and the effluent concentrations are converted to logarithms. The relationship between the Probits and the logarithmic values of the concentrations is approximately linear. A Probit regression line drawn through the data points is used to estimate the LC50 and its precision. NOTE: TO USE PROBIT ANALYSIS, AT LEAST TWO PARTIAL MORTALITIES MUST BE OBTAINED IN THE TOXICITY TEST. The following steps are involved in the analysis:

Step 1. Prepare a table (Table 17) with the following headings:

- a. Effluent concentrations used in the test (%).
- b. $\log_{10}$ of the effluent concentration (x).
- c. Number of organisms at each concentration (N).
- d. Observed mortality at each concentration (r).
- e. Percent mortality at each concentration (P).
- f. Empirical (observed) Probit (EP)
- g. Expected (calculated) Probit (Y)

Step 2. Items "a-e" consist of data from the experiment.

Step 3. The EP is obtained from Table 18 by entering P.

Step 4. A graph (Fig. 13) is prepared using the $\log_{10}$ concentrations (x) and the EPs. A regression line is then drawn through the points by eye.

Step 5. From the regression line, determine the $\log_{10}$ concentration (m), or $\log_{10}$ LC50, corresponding to a Probit of 5.0. In our example, $m = 1.72$.

Step 6. From the regression line, determine "s", the rate of increase in $\log$ concentration (x) per unit increase in the empirical probit (EP) as follows: from the points at $x = 0.80$ and $x = 2.00$ we obtain probits of 3.30 and 5.55 respectively. The slope is $(2.00 - 0.80)/(5.55 - 3.30)$, which equals $1.20/2.25$, or 0.533, the value of "s".

Step 7. The values of Y can now be calculated from $Y = 5 + (x - m)/s$, and entered in Table 17.

Step 8. To test the hypothesis of no association between effluent concentration and Probit response, we construct Table 19. The quotients in the last column are summed, and the sum is a χ^2 statistic with degrees of freedom equal to two less than the number of effluent levels used in the probit analysis ($5 - 2 = 3$). A significant χ^2 value implies that the

TABLE 17. TOXICITY DATA FOR PROBIT ANALYSIS

Effl Conc (%)	Log10 Conc (x)	Number of Organisms (N)	Observed Mortality (r)	% Mortality (P)	Empirical Probit (EP)	Calculated Probit (Y)
100	2.00	20	15	75	5.67	5.55
50	1.70	20	9	45	4.87	4.96
25	1.40	20	5	25	4.33	4.38
12.5	1.10	20	2	10	3.72	3.79
6.25	0.80	20	1	5	3.36	3.30

TABLE 18. EMPIRICAL (OBSERVED) PROBIT PERCENT MORTALITY RELATIONSHIP

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
%	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99a	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

a. Values between 99.0 and 99.9

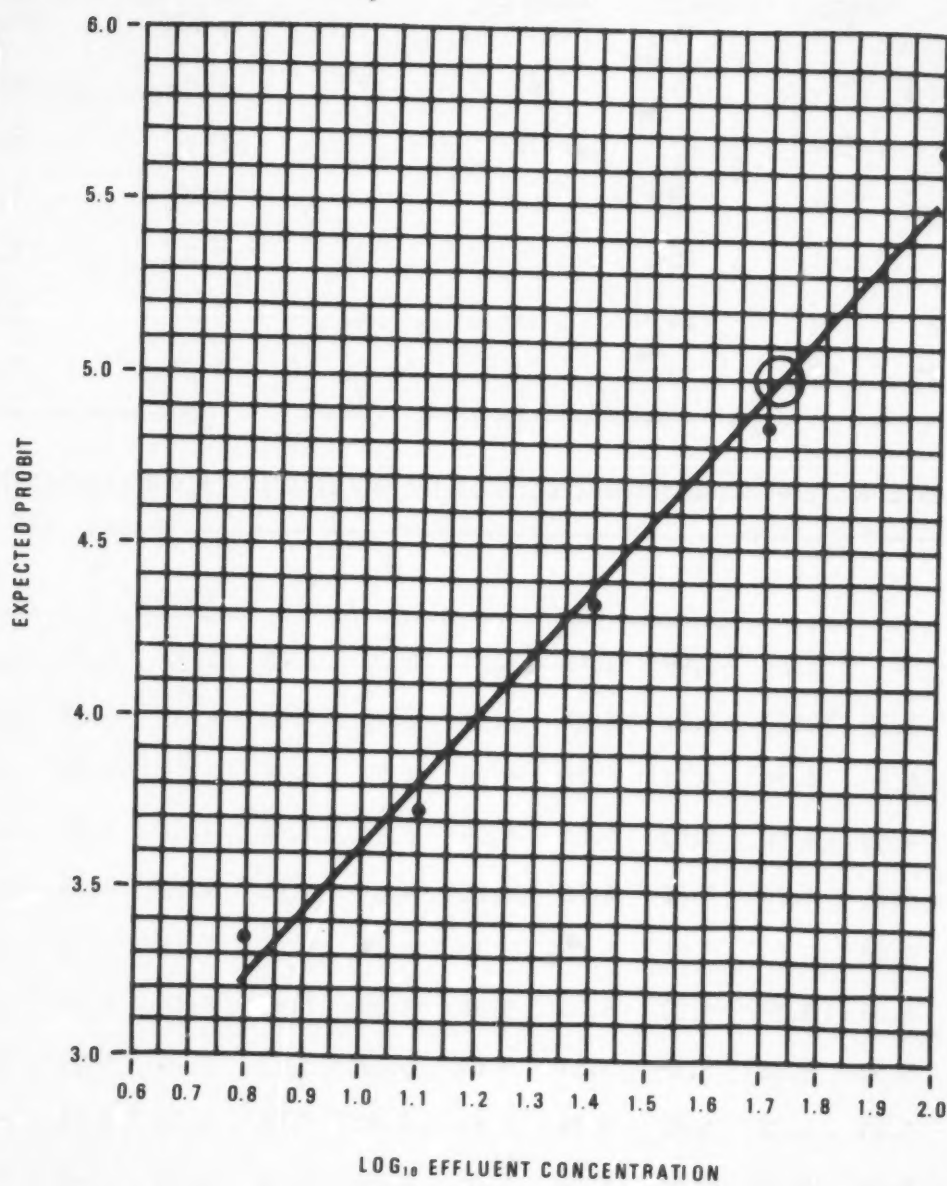


Figure 13. Plot of toxicity test data for Probit analysis.

data are not adequately represented by the regression line. In our example, the Chi-square value equals 0.321 (Table 19), which is not significant.

Step 9. To determine the standard error for the $\log_{10}$ LC50 (m) estimate obtained above from the regression line, we construct Table 20, with the headings as follows:

- Log₁₀ of the effluent concentration (x).
- Number of organisms at each concentration (N).
- Expected, or calculated, probit (Y).
- Weighting factor (w), obtained by entering Table 21 with the expected probit (Y).
- Products (Nw), (Nwx), and (Nwx²) are obtained as the last three columns. The sums of these columns have the prefix "S", (SNw, SNwx, and SNwx²).

An approximate value for the standard error (SE) of $\log_{10}$ LC50 is calculated as follows using "s", the rate of increase in log concentration (x) per unit increase in the empirical probit (EP) computed above, where $s = 0.553$ and $SNw = 46.62$:

$$SE (\log_{10} LC50) = \frac{s}{(SNw)^{0.5}} = \frac{0.553}{(46.62)^{0.5}} = 0.0781$$

- Using the standard error of the $\log_{10}$ LC50 derived in Paragraph "a" above, the approximate value for SE LC50 can be obtained by raising 10 to the power of m (which is $10^{1.72} = 52.5$), multiplying by $\log_{10}$ (which is 2.3026) and then by SE $\log_{10}$ LC50 (which is 0.0781). Thus:

$$SE LC50 = 52.5 \times 2.3026 \times 0.0781 = 9.43$$

- A more accurate estimate of SE $\log_{10}$ LC50 is obtained from the information in Table 17 as follows.

$$SE \log_{10} LC50 = \left[s^2 \left\{ \frac{1}{SNw} + \frac{(m - x)^2}{SNw(x - x)^2} \right\} \right]^{0.5}$$

Where (from Table 20):

$$\begin{aligned} x &= 71.21/46.62 = 1.527 \\ m &= 1.72 \\ SNw &= 46.62 \\ SNwx &= 71.72 \\ SNwx^2 &= 115.40 \\ SNw(x-x)^2 &= SNwx^2 - (SNwx)^2/SNw \\ &= 115.40 - (71.21)^2/46.62 \\ &= 6.630. \end{aligned}$$

Table 19. CHI-SQUARE DETERMINATION.

% Effl. Conc.	Log10 Conc. (x)	Expected or Calculated Probit (Y)	Expected % (P)	Number of Organisms (N)	Observed Mortality (r)	Expected Mortality (NP)	Deviations in Mortality (r-NP)	Contribution to Chi-square $\frac{(r-NP)^2}{NP}$
100	2.00	5.55	71.0	20	15	14.2	0.8	0.64/4.12 = 0.155
50	1.70	4.96	48.5	20	9	9.7	-0.7	0.49/4.99 = 0.098
25	1.40	4.38	26.7	20	5	5.3	-0.3	0.09/3.88 = 0.023
12.5	1.10	3.79	11.5	20	2	2.3	-0.3	0.09/2.04 = 0.044
6.25	0.80	3.30	4.5	20	1	0.9	0.1	0.01/8.60 = 0.001
								0.321

TABLE 20. STANDARD ERROR OF THE LOG₁₀ LC50 (M).

Log ₁₀ Conc (x)	Number of Organisms (N)	Calculated Probit (Y)	Weight (w)	Product (Nw)	Product (Nwx)	Product (Nwx ²)
2.00	20	5.55	0.569	11.38	22.76	45.52
1.70	20	4.96	0.635	12.70	21.59	36.70
1.40	20	4.38	0.552	11.04	15.46	21.64
1.10	20	3.79	0.367	7.34	8.07	8.88
0.80	20	3.30	0.208	<u>4.16</u>	<u>3.33</u>	<u>2.66</u>
SUM ("S") ^a				46.62	71.21	115.40

a. See paragraph 9(e), above.

TABLE 21. WEIGHTING FACTOR (W) FOR THE CALCULATED PROBIT (Y).

Y	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1	0.001	0.001	0.001	0.002	0.002	0.003	0.005	0.006	0.008	0.011
2	0.015	0.019	0.025	0.031	0.040	0.050	0.062	0.076	0.092	0.110
3	0.131	0.154	0.180	0.208	0.238	0.269	0.302	0.336	0.370	0.405
4	0.439	0.471	0.503	0.532	0.558	0.581	0.601	0.616	0.627	0.634
5	0.637	0.634	0.627	0.616	0.601	0.581	0.558	0.532	0.503	0.471
6	0.439	0.405	0.370	0.336	0.302	0.269	0.238	0.208	0.180	0.154
7	0.131	0.110	0.092	0.076	0.062	0.050	0.040	0.031	0.025	0.019
8	0.015	0.011	0.008	0.006	0.005	0.003	0.002	0.002	0.001	0.001

Thus:

$$\begin{aligned} \text{SE Log}_{10} \text{LC50} &= \left[(0.533)^2 \left\{ \frac{1}{46.62} + \frac{(1.72 - 1.527)^2}{6.630} \right\} \right]^{0.5} \\ &= (0.00764)^{0.5} = 0.0874. \end{aligned}$$

This result gives rise to a more accurate approximate value for SE LC50, by substituting the more accurate value of SE log₁₀ LC50 in the previous expression. In our example, SE LC50 = (52.5) (2.3026) (0.0874) = 10.55.

The LC50 $\pm$ SE = 52.5% $\pm$ 10.55.

COMPUTER CALCULATION OF LC50s and CONFIDENCE INTERVALS

Computer programs are available from EMSL-Cincinnati for the following analyses:

1. Binomial
2. Moving average
3. Moving average-angle
4. Probit
5. Trimmed Spearman-Kärber

The results obtained from these programs differ only slightly in most cases, as shown in Table 22, and the LC50s are similar to those obtained with the graphical method.

Listings for the above programs, together with instructions and sample data for their use, are included in Appendix E.

These programs also reside on the EPA IBM 370/168 at Research Triangle Park (NCC). Instructions for the direct transfer of machine readable copies of the programs to other computers (such as PDP-11/70s) may be obtained by contacting the Aquatic Biology Section, EMSL-Cincinnati.

COMPARISON OF LC50s AND 95% CONFIDENCE LIMITS OBTAINED WITH DIFFERENT COMPUTER PROGRAMS AND THE GRAPHICAL METHOD. a,b,c

a. Prepared by James Dryer, ENSL-Cincinnati
b. Twenty (20) organisms per treatment.
c. where: A = Moving Average-angle
B = Probit analysis
C = Trimmed Spearman-Kärber
D = TOXSTAT (Program developed by ERL-Duluth)
Da = Binomial test
Db = Moving average
Dc = Probit analysis
E = Graphical Method

UL = Upper Confidence Limit
LL = Lower Confidence Limit

SECTION 12

ESTIMATING THE POTENTIAL FOR ACUTE AND CHRONIC TOXICITY OF WASTE IN RECEIVING WATERS BASED ON THE LC50 FROM ACUTE TOXICITY (LETHALITY) TESTS

(Prepared by Lee B. Tebo)

To avoid the potential for toxicity in receiving waters, the following relationship must hold:

$$IWC \leq NOEC \quad \text{(Formula 1)}$$

Where: NOEC = No observed effect concentration, expressed as percent waste.

IWC = Instream (receiving water) waste concentration, expressed in the same unit.

Several critical considerations are embedded in Formula 1 above:

1. Is the NOEC to be expressed in terms of acute toxicity (lethality) or chronic toxicity, or both?
2. If the LC50s from acute toxicity (lethality) tests described in this manual are to be used to estimate the acute or chronic NOECs in receiving waters, there must be known relationships between the LC50 and the acutely lethal or chronically toxic concentrations. These relationships may be expressed in the state water quality standards.
3. A margin of safety, based on varying species sensitivity, should be provided. The need for a margin of safety may be minimized by testing a diverse representation of vertebrate and invertebrate species and using the LC50 of the most sensitive species.
4. What design flow (7Q10, 30Q2, etc.) is to be used in calculating the IWC in rivers and streams? (7Q10 is lowest average daily flow during any consecutive seven days in any ten-year period; 30Q2 is the lowest average daily flow for consecutive 30 days in any given two-year period).

In the following discussion of the principles involved in the use of the LC50s from acute toxicity (lethality) tests to estimate the acute or chronic NOECs in receiving waters, the application factors, design flows, and margins of safety employed are used only as examples, and do not necessarily represent current Agency policy or guidance. The values used for specific discharges should be based on the existing water quality standards, Agency policy and/or guidance (USEPA, 1984b), where applicable, and current scientific knowledge, and should be applied on a case-by-case basis.

ESTIMATING THE ACUTE NOEC

To employ the acute LC50 to protect the receiving water against acutely toxic (lethal) concentrations of waste, the use of an (acutely lethal concentration)/(acute LC50) ratio of 1:3 has been suggested (Hart et al., 1945; Tebo, 1985). If this ratio is used, Formula 1 becomes:

$$IWC \leq \frac{LC50}{3} \quad (\text{Formula 2})$$

and
$$3(IWC) \leq LC50 \quad (\text{Formula 3})$$

The generalized form of Formula 3 is:

$$\frac{IWC}{AF} \leq LC50 \quad (\text{Formula 4})$$

Where: AF = An application factor which is the ratio of the acute or chronic toxicity (NOEC) to the acute LC50, as described above.

For rivers and streams:

$$IWC = \frac{Q_w}{(Q_w + Q_r)} \times 100 = \frac{100(Q_w)}{(Q_w + Q_r)} \quad (\text{Formula 5})$$

Where: Q_w = Waste flow in a standard unit, such as mgd.
 Q_r = Receiving water flow (design stream flow) in the same standard unit.

To protect rivers and streams from acute lethality (acute NOEC), using the example of an acutely lethal concentration/acute LC50 ratio of 1:3, Formula 3 above becomes:

$$\frac{300(Q_w)}{(Q_w + Q_r)} \leq LC50 \quad (\text{Formula 6})$$

The design stream flow (Q_r) for the above determination may be specified in the state water quality standards or may be based on a determination of the frequency that the regulatory agency is willing to allow acutely toxic (lethal) conditions in the receiving waters. For a first approximation, the 7Q10 is frequently used, and indeed this is the design flow presently specified in many state water quality standards.

The remaining consideration concerns the margin of safety to be provided to account for varying species sensitivity. If the LC50 is based on the most sensitive of two species tested (an invertebrate and vertebrate), a safety margin of one order of magnitude is suggested as a reasonable approximation.

Thus, after including a margin of safety of one order of magnitude for variations in species sensitivity, Formula 6 becomes:

$$\frac{3000(Q_w)}{(Q_w + Q_r)} \leq LC50 \quad (\text{Formula 7})$$

ESTIMATING THE CHRONIC NOEC

The IWC for protecting rivers and streams from chronic toxicity (chronic NOEC) is derived using Formulas 2 - 7 in the same manner as described above for the acute NOEC. For protection against chronically toxic concentrations of waste in receiving waters, the National Academy of Sciences (USEPA, 1973) proposed the use of (chronically toxic concentration)/(acute LC50) ratios of 1:20 for nonpersistent wastes and 1:100 for persistent wastes. If these chronic ratios are used, depending on the nature of the waste, Formula 2 becomes:

$$IWC \leq \frac{LC50}{20} \quad \text{or,} \quad IWC \leq \frac{LC50}{100} ,$$

and Formula 7 becomes:

$$\frac{20,000(Q_w)}{(Q_w + Q_r)} \leq LC50 \quad \text{or,} \quad \frac{100,000(Q_w)}{(Q_w + Q_r)} \leq LC50$$

It is important to recognize that if sampling is inadequate to account for short term (diel) and long term (annual) variations in waste toxicity, an appropriate safety factor must be applied to the formulas discussed above (USEPA, 1984b).

LAKES, IMPOUNDMENTS, AND ESTUARIES

Formula 4 may also be used where a mixing zone is specified. In the case of lakes, impoundments, and estuaries, a mixing zone must be provided or receiving water toxicity calculations cannot be performed. By definition, a mixing zone includes a boundary around the outfall within which there should be no acutely lethal conditions and outside of which the waste should not exhibit acute or chronic toxicity. Where a mixing zone is to be used, there are three steps involved in establishing safe receiving water concentrations:

1. Establish mixing zone boundaries according to state water quality standards.
2. Determine the waste concentration at designated mixing zone boundaries. This can be determined using an appropriate model at design flow conditions, or determined empirically by conducting a dye study.
3. Calculate the potential for acute or chronic toxicity using Formula 4. The IWC in Formula 4 is the waste concentration at the appropriate mixing zone boundary.

If no mixing zone is permitted, the IWC in Formula 4 is equivalent to 100% effluent (effluent at the end of the pipe).

SECTION 13

REPORT PREPARATION AND DATA UTILIZATION

The report should contain the following types of information. The specific content and format of the report, however, may vary on a case-by-case basis.

1. Introduction - Objectives, how the advance arrangements were made for the test, persons contacted (state, industry, etc.), names of investigators, plant location and NPDES permit number, the name of the receiving water body, and the date the test was conducted.
2. Plant Operations - Type of plant, operating schedule, final products, schematic of waste treatment, discharge information, flows, etc.
3. Effluent and Dilution Water - Sources and Sampling Methods -
 - a. A detailed description of the effluent, including its source, date and time of collection, composition, known physical and chemical properties, and variability.
 - b. The source of the dilution water, the date and time of its collection, its chemical characteristics, and a description of any pretreatment.
4. Test Methods - Description of methods and any deviations from established methods
 - a. A description of the test procedure, the definition of the adverse effect (death, immobility, etc.) used in the test, a description of the test chambers, including the depth and volume of solution, the way the test was begun, the number of organisms per treatment, and the loading. For the flow-through system, indicate the water volume changes per 24 h in each test chamber.
 - b. Detailed information about the test organisms, including scientific name, mean length and weight (where appropriate), age, life stage, source, history, observed diseases, treatments, and acclimation procedure.

- c. The methods used for, and the results of, all chemical analyses.
- d. The mean and range of the acclimation temperature and the test temperature.

5. Results - Should include the following:

- a. The results of all chemical analyses of the effluent and dilution water.
- b. A daily record of the number and percentage of organisms in each test chamber (including the control chambers) that died or showed the selected "effect."
- c. A summary of general observations of other effects or symptoms of toxicity observed during the test.
- d. An LC50 value for the test organisms for the period of interest. If 100% effluent did not kill or affect 50% or more of the test organisms, report the percentage of the test organisms killed or affected by various concentrations of the effluent.
- e. When possible, the 95% confidence interval for the LC50, and the method used to calculate it.
- f. Any deviation from the standard test methods.
- g. Any other relevant information.

6. Discussion and Recommendations -

- a. Prediction of potential acute and chronic toxicity in the receiving water, based on the IWC method described in Section 12.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

1. DAPHNIA (D. MAGNA AND D. PULEX)¹

GEOGRAPHICAL AND SEASONAL DISTRIBUTION

D. magna is principally a lake dweller and is restricted to waters in northern and western North America exceeding a hardness of 150 mg/L (as CaCO₃) (Pennak, 1978). D. pulex (Fig. 14) is found over most of the North American continent. It is principally a pond dweller, but also is found in lakes.

Daphnia populations generally are sparse in winter and early spring, but as the water temperature reaches 6° to 12°C, they increase in abundance and subsequently may reach population densities as high as 200 to 500 individuals/L (Pennak, 1978). Populations in ponds decline during the summer months to very low numbers. In the autumn there may be a second population pulse, followed by a decline to winter lows.

During most of the year populations of Daphnia consist almost exclusively of females, the males being abundant only in spring or autumn. Males are distinguished from females by their smaller size, larger antennules, modified postabdomen, and first legs, which are armed with a stout hook used in clasping. Production of males appears to be induced principally by high population densities and subsequent accumulations of excretory products, and/or a decrease in available food. These conditions may induce the appearance of sexual (resting) eggs in cases or ephippia (Fig. 15), which are cast off during the next molt. It appears that the shift towards male and sexual egg production is related to the metabolic rate of the parent. Any factor, in addition to those above, which tends to lower metabolism may be responsible. As a rule, males and ephippia will not be observed in the lab unless stock cultures are neglected, or the culture experiences some type of environmental stress.

LIFE CYCLE

The life span of Daphnia, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the species and environmental conditions (Pennak, 1978). Generally the life span increases as temperature decreases, due to lowered metabolic activity. The average life span of D. magna is about 40 days at 25°C, and about 56 days at 20°C. The average life span of Daphnia pulex at 20°C is approximately 50 days. Four distinct periods may be recognized in the life history of Daphnia: (1) egg, (2) juvenile, (3) adolescent, and (4) adult (Pennak, 1978). Typically, a clutch of 6 to 10 eggs is released into the brood chamber. The eggs hatch in the brood chamber and

¹Taken from Weber and Peltier, 1981.

the juveniles, which are already similar in form to the adults, are released in approximately two days when the female molts (casts off her exoskeleton or carapace). The time required to reach maturity (produce their first offspring) varies from six to 10 days and also appears to be dependent on body size. The growth rate of the organism is greatest during its juvenile stages (early instars), and the body size may double during each of these stages. D. pulex has three to four juvenile instars, whereas D. magna has three to five instars. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

Following the juvenile stages, the adolescent period is very short, and consists of a single instar. It is during the adolescent instar that the first clutch of eggs reaches full development in the ovary. Generally, eggs are deposited in the brood chamber within minutes after molting, and the young which develop are released just before the next molt.

D. magna usually has six to 22 adult instars, and D. pulex has 18 to 25. In general, the duration of instars increases with age, but also depends on environmental conditions. A given instar generally lasts approximately two days under favorable conditions, but when conditions are unfavorable, it may last as long as a week.

Four events take place in a matter of a few minutes at the end of each adult instar: (1) release of young from the brood chamber to the outside, (2) molting, (3) increase in size, and (4) release of a new clutch of eggs into the brood chamber. The number of young per brood is highly variable for Daphnia, depending primarily on food availability and environmental conditions. D. magna and D. pulex may both produce as many as 30 young during each adult instar, but more commonly the number is six to 10. The number of young released during the adult instars of D. pulex reaches a maximum at the tenth instar, after which there is a gradual decrease (Anderson and Zupancic, 1937). The maximum number of young produced by D. magna occurs at the fifth adult instar, after which it decreases (Anderson and Jenkins, 1942).

MORPHOLOGY AND TAXONOMY

D. pulex attains a maximum length of approximately 3.5 mm, whereas D. magna are much larger, and attain a length of 5.0 or 6.0 mm. However, these two species can be differentiated with certainty only by determining the size and number of spines on the postabdominal claws (Fig. 16), using a dissecting or compound microscope (Pennak, 1978).

CULTURING METHODS

1. Sources of Organisms

Daphnia are available from the Environmental Monitoring & Support Laboratory-Cincinnati, from other EPA, state or private laboratories in which Daphnia are used in toxicity tests, and from commercial biological supply houses. Only a small number of organisms (20-30) are needed to

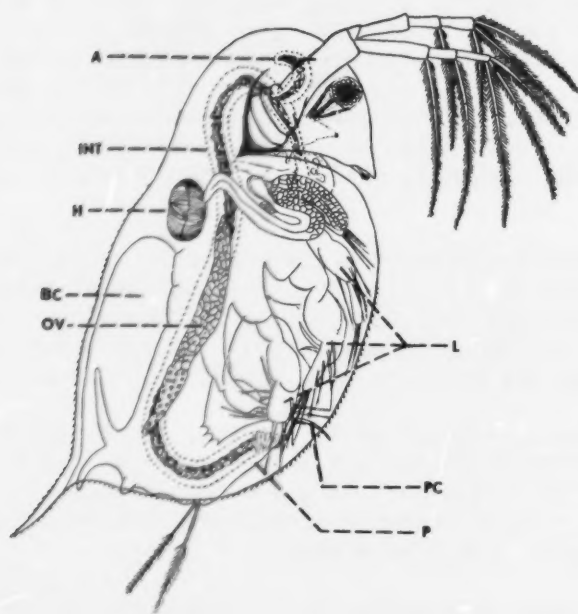


Figure 14. Anatomy of female *Daphnia pulex* (De Geer), X70; A, antenna; BC, brood chamber; H, heart; INT, intestine; L, legs; OV, ovary; P, postabdomen; PC, postabdominal claw. (From Pennak, 1978).

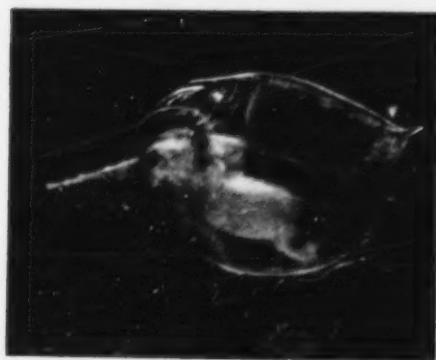


Figure 15. *Daphnia magna* with ephippium (sexual egg case). (From Doma, 1979).

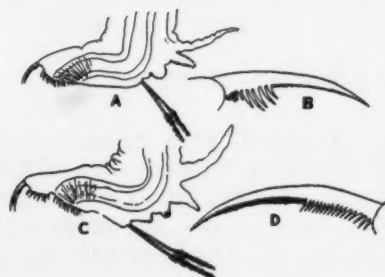


Figure 16. *Daphnia* postabdomin. *Daphnia pulex*: A, postabdomen; B, postabdominal claw. *Daphnia magna*: C, postabdomen; D, postabdominal claw. (From Pennak, 1978)

start a culture. D. pulex is preferred over D. magna by some biologists because it is more widely distributed than D. magna, and is easier to culture. However, the neonates of D. magna are larger and, therefore, somewhat easier to use in toxicity tests. Guidance on the source and species of Daphnia to be used by a permittee for effluent toxicity tests should be obtained from the permitting authority, and the species used should be verified by a taxonomic authority.

2. Culture Media

Although Daphnia stock cultures can be successfully maintained in some tap waters, well waters, and surface waters, use of synthetic water as the culture medium is recommended because (1) it is easily prepared, (2) it is of known quality, (3) it produces predictable results, and (4) allows adequate growth and reproduction. Reconstituted hard water (hardness: 160 to 180 mg/L CaCO_3) is recommended for D. magna, whereas moderately hard reconstituted water (hardness: 80 to 90 mg/L CaCO_3) is recommended for D. pulex.

The preparation of the media is as follows:

a. Hard reconstituted water medium (for D. magna)

<u>Reagent</u>	<u>Concentration (mg/L)</u>
NaHCO_3	192.0
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	120.0
MgSO_4	120.0
KCl	8.0

b. Moderately-hard reconstituted water medium (for D. pulex)

<u>Reagent</u>	<u>Concentration (mg/L)</u>
NaHCO_3	96.0
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	60.0
MgSO_4	60.0
KCl	4.0

The compounds are dissolved in distilled or deionized water and the media are vigorously aerated for several hours before using. The initial pH of the media is approximately 8.0, but it will rise as much as 0.5 unit as the Daphnia population increases. Although Daphnia can survive over a wide pH range, the optimum range is 7.0 to 8.6 (Lewis and Weber, 1985). Since the pH usually remains within this range, there is no need to monitor or adjust it.

To make up media as needed (usually once a week for each set of five stock cultures), prepare stock solutions of KCL, $MgSO_4$ and $NaHCO_3$ as follows (these solutions will provide a six to 12 months supply):

KCL Stock Solution:

- a. Place 8 g of crystalline, reagent grade KCL in a 1-L volumetric flask.
- b. Bring the volume to 1 L with distilled water.
- c. Store in a 1-L polyethylene bottle.

MgSO₄ Stock Solution:

- a. Place 120 g of reagent grade, anhydrous $MgSO_4$ powder in a 1-L volumetric flask.
- b. Bring the volume to 1 L with distilled water.
- c. Store in a 1-L polyethylene bottle.

NaHCO₃ stock solution:

- a. Place 96 g reagent grade $NaHCO_3$ powder in a 1-L volumetric flask.
- b. Bring the volume to 1 L with distilled water.
- c. Store in a 1-L polyethylene bottle.

To make hard reconstituted water for Daphnia magna:

- a. Place 18.2 L of deionized or distilled water in a 20-L carboy that has been thoroughly cleaned (see Section 4 on cleaning glassware).
- b. Add 2.4 g of $CaSO_4 \cdot 2H_2O$ to 1 L of deionized or distilled water in a 2-L beaker, and stir until dissolved. Add this solution to the carboy and mix well.
- c. Add each of the following stock solutions to the carboy and mix well after each addition:
 - (1) 20 mL KCL stock solution
 - (2) 20 mL $MgSO_4$ stock solution
 - (3) 40 mL $NaHCO_3$ stock solution.
- d. Vigorously aerate the medium in the carboy for 2 h.

To make moderately-hard reconstituted water for Daphnia pulex:

- a. Place 18.6 L of deionized or distilled water in a 20-L carboy which has been thoroughly cleaned (according to instructions in Section 4).
- b. Add 1.2 g of $CaSO_4 \cdot 2H_2O$ to 1 L of deionized or distilled water in a 2-L beaker and stir until dissolved. Add this solution to the carboy.

c. Add each of the following stock solutions to the carboy and mix well after each addition:

- (1) 10 mL KCL stock solution
- (2) 10 mL MgSO_4 stock solution
- (3) 20 mL NaHCO_3 stock solution

d. Vigorously aerate the medium in the carboy 2 h.

3. Feeding: Quantity and Frequency

Food preparation and feeding are of great importance in Daphnia culturing. Daphnia can be cultured using algae or a prepared food consisting of a suspension of a trout chow, alfalfa, and yeast. The latter diet is easily prepared and provides adequate nutrition for organisms used in acute toxicity tests (Winner et al., 1977).

Trout chow, alfalfa, and yeast:

The food is prepared as follows:

- a. Place 6.3 g of trout chow pellets, 2.6 g of dried yeast, and 0.5 g of dried alfalfa in a blender.

NOTE: The trout chow must conform to Fish & Wildlife Service Specification PR(11)-78, and can be obtained through livestock feed stores. Dried yeast, such as Fleischmann's dried yeast, can be obtained at any grocery store. Dried alfalfa can be obtained at health food stores.

- b. Add 500 mL of distilled or deionized water.
- c. Mix at high speed for 5 min.
- d. Place in a refrigerator and allow to settle for 1 h.
- e. Decant the top 300 mL and save; discard the remainder.
- f. Place 30 to 50 mL aliquots in small (50 to 100 mL) polyethylene bottles with screw caps and freeze.
- g. Thaw portions as needed. After thawing, keep in refrigerator for a maximum of one week, then discard.

Feed 1.5 mL of prepared food per 1000 mL of medium, three times per week (i.e. Monday, Wednesday, Friday). There may be some excess food in the medium at this rate of feeding, but if the medium is aerated continuously and replaced each week, as discussed below, this should cause no problems.

Algae:

An alternate method^a of feeding Daphnia involves the use of Selenastrum capricornutum Printz, the green alga commonly employed in the

^aBased on information provided by Dr. Thomas Waller and James Lazorchak, Environmental Sciences Program, University of Texas at Dallas, P.O. Box 688, MS BE 22, Richardson, Texas 75080 (214-690-2966 or 2970).

algal growth potential test. The algal cultures are maintained as described in Miller et al. (1978). Algal stock cultures are started each Monday, Wednesday, Friday and Sunday. The objectives of the culture procedure are to produce seven-day old cultures that contain four to five million algal cells per mL, and two to four day old cultures that contain one to three million cells per mL. The algal food is prepared and fed Monday, Wednesday, and Friday, as described below:

- a. Volumes of seven-day-old and three-day-old algal cultures are combined in a ratio of two volumes to one, respectively (for example, 1000 mL seven-day-old culture and 500 mL of three-day-old culture.
- b. The algae are sedimented by centrifugation and resuspended in a volume of moderately hard or hard reconstituted water (such as used for the Daphnia cultures) equal to the original volume of seven-day-old algal cultures from which the algae were concentrated.
- c. The resuspended algae should number three to four million cells per mL. A sufficient volume of the cell suspension is added to the Daphnia stock cultures every day to provide approximately 100,000 algal cells per mL of Daphnia culture. This would involve the addition of approximately 25 mL of cell suspension to 1 L of Daphnia stock culture.

4. Culture Temperature

Daphnia can be cultured successfully over a wide range of temperatures, but should be protected from sudden changes in temperature, which may cause death. The optimum temperature is approximately 20°C, and if ambient laboratory temperatures remain in the range of 18 to 26°C, normal growth and reproduction of Daphnia can be maintained without special temperature control equipment.

5. Illumination

The variations in ambient light intensities (50 to 100 ft c) and prevailing day/night cycles in most laboratories do not seem to affect Daphnia growth and reproduction significantly. However, a minimum of 16 h of illumination should be provided each day.

6. Culture Vessels

Culture vessels of clear glass are recommended since they allow easy observation of the Daphnia. A practical culture vessel is an ordinary 3-L glass beaker, which can be filled with approximately 2.75 L of medium (reconstituted water). Maintain several (at least five) culture vessels, rather than only one. This will ensure back-up cultures so that in the event of a population "crash" in one or several chambers, the entire

Daphnia population will not be lost. If a 3-L vessel is stocked with 30 Daphnia, it will provide approximately 300 young each week.

Initially, all culture vessels should be washed well (see Section 5). After the culture is established, clean each chamber weekly with distilled or deionized water and wipe with a clean sponge to rid the vessel of accumulated food and dead Daphnia (see section on culture maintenance below). Once per month wash each vessel with detergent during medium replacement. Rinse three times with tap water and then with culture medium to remove all traces of detergent.

7. Aeration

Daphnia can survive when the dissolved oxygen concentration is as low as 3 mg/L, but do best when the level is above 6 mg/L. Each culture vessel should be continuously and gently aerated. This is best accomplished by using air stones. The air can come from either an aquarium air pump or from a general laboratory compressed air supply. If the laboratory air supply is used, first pass it through a flask full of cotton batting to filter out oil or other contaminants.

8. Weekly Culture Media Replacement

Careful culture maintenance is essential. The medium in each stock culture vessel should be replaced each week with fresh medium. This is best accomplished as follows:

- a. Siphon the old medium out, using plastic tubing (6 mm) covered with fine mesh netting around the open end.
- b. Retain 1/10 (300 mL) of the original medium containing the Daphnia population.
- c. Pour this (old) medium containing the Daphnia into a temporary holding vessel.
- d. Clean the culture vessel as described above.
- e. Fill the newly-cleaned vessel with fresh medium. Gently transfer (by pouring) the contents of the temporary holding vessel (old medium and Daphnia) into the vessel containing the fresh medium.

If the medium is not replaced weekly, waste products will accumulate, which could cause a population crash or the production of males and/or sexual eggs.

Daphnia populations should be thinned weekly to about 30 adults per stock vessel to prevent over-crowding, which may cause a population crash, or the production of males and/or ehippia. A good time to thin the populations is during medium replacement. To transfer Daphnia use a 15-cm disposable, jumbo bulb pipette, or 10-mL "serum" pipette that has had the delivery end cut off and fire polished. The diameter of the opening should be approximately 5 mm. When using a serum pipette, a pipette bulb, such as

a Propipette^R, or (Mopet^R) portable, motorized pipetter, provides the controlled suction needed when selectively collecting Daphnia.

Liquid containing adult D. pulex and D. magna can be poured from one container to another without risk of air becoming trapped under their carapaces. However, the very young Daphnia are much more susceptible to air entrapment and for this reason should be transferred from one container to another using a pipette. The tip of the pipette should be kept under the surface of the liquid when the Daphnia are released.

Each culture vessel should be covered with a clear plastic sheet or glass plate to exclude dust and dirt and minimize evaporation.

TEST ORGANISMS

Daphnia magna or Daphnia pulex 24 h or less in age (neonates, or first instars) are to be used in the tests. To obtain the necessary number of young for a test, remove adult females bearing embryos in their brood pouches from the stock cultures 24 h preceding the initiation of the test, and place them in 400 mL beakers containing 300 mL of medium and 0.5 mL of prepared food (see culturing method above). The young that are found in the beakers the following day are used for the toxicity test. Five beakers, each containing 10 adults, usually will supply enough first instars for one toxicity test.

Since the appearance of ephippia in cultures generally is indicative of unfavorable conditions, Daphnia used for toxicity tests should not be taken from cultures that are producing ephippia.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

2. MYSIDS (MYSIDOPSIS BAHIA)¹

DISTRIBUTION

Mysids (Fig. 17) are primarily coastal water organisms and occur in estuaries along the Atlantic, Gulf and Pacific coasts. They are an important link in estuarine and marine food chains and serve as the primary food for many species of fish (Molenock, 1969; Shaefer, 1970; Jacobs and Grant, 1974; Nimmo et al., 1977; Price, 1982). Mysidopsis almyra, M. bahia, M. bigelowi, Metamysidopsis elongata, and Neomysis americana have been used extensively in toxicity tests (Nimmo and Hamaker, 1982). The mysids have been called "opossum shrimp" because the female carries its young in a brood pouch while they develop.

LIFE CYCLE

In laboratory studies, it has been observed that females of M. bahia reach sexual maturity in 12 to 20 days, depending on water temperature and diet (Nimmo et al., 1977). Unlike Daphnia, the eggs will not develop unless fertilized. Brood pouches form when the female reaches the age of 13 to 15 days, and young are released in 17 to 20 days. The number of eggs produced is a direct function of body length. Mature females may produce as many as 20 young per brood, but average five to seven. A new brood is produced every four to seven days. The juveniles are planktonic for the first 24 h and then settle to the bottom, orient to the current, and actively pursue food organisms such as Artemia. Carr et al. (1980) reported that the stage in the life cycle of M. almyra most sensitive to drilling mud was the first juvenile molt, which occurs between 24 and 48 h after release from the brood pouch.

MORPHOLOGY AND TAXONOMY

Adults of M. bahia range in length from 4.4 mm to 9.4 mm (Molenock, 1969), measured from the anterior margin of carapace to the end of uropods. Living organisms are usually transparent, but may be tinted yellow, brown or black. Mysidopsis bahia differs from the other two species of Mysidopsis (M. almyra and M. bigelowi) by the armature of the telson, the spines on the uropod, and an unsegmented antennal scale (Molenock, 1969)(Figs. 17 and 18).

¹Taken from Weber and Peltier, 1981.

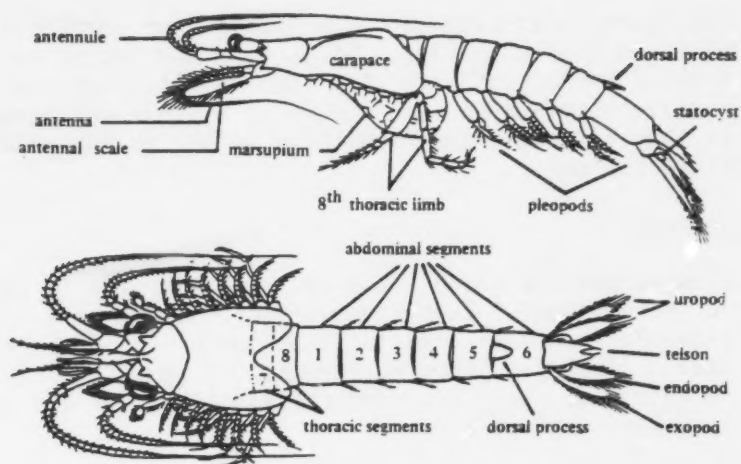


Figure 17. Lateral and dorsal view of a typical mysid. (From Stuck et al., 1979).

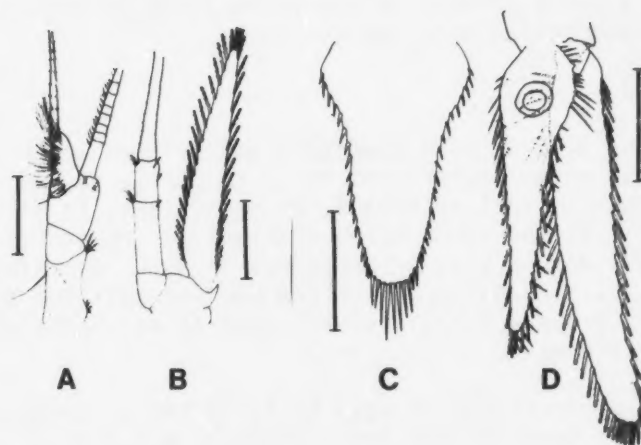


Figure 18. Morphological characteristics used in mysid identification (*Mysidopsis bahia*). A, antenna 1, ventral male; B, antenna 2; C, telson; D, right uropod, dorsal. Scale lines 0.5 mm in length. (From Molenock, 1969).

CULTURE METHODS

1. Sources of Organisms

Mysids can be obtained from EPA laboratories, or commercial sources, or can be collected from estuaries. Since many species of mysids may be present at a given collection site, the identification of the organisms selected for culture should be verified by an experienced taxonomist. The permittee should consult the permitting authority for guidance on the source of test organisms.

2. Culture Vessels

Stock cultures are maintained in a recirculating static system in 200-L aquaria (Ward, 1984). Each aquarium is equipped with a commercially-available, under-gravel filter which is covered with two to five cm of small, smooth, crushed oyster-shell fragments (four to 15 mm in length) or dolomite. Both are commercially available and should be washed and autoclaved before use.

The culture medium (natural or synthetic sea water) is added to the aquarium containing the shells and conditioned (aged) for two to three weeks before mysids are introduced. During this period the water is circulated through the under-gravel filter.

The tops of the two bubble-up standpipes from the filters are placed immediately above the water surface. The rate of water flow through the filters should be sufficient to establish a moderate current in the aquarium. This will be evident in the orientation of the mysids, which commonly align themselves with the current.

3. Culture Media

Use filtered (0.45 μ m pore diameter) natural sea water or reconstituted sea water prepared at 20 to 25 ppt by adding artificial marine salts, such as Instant Ocean^R, or equivalent, to distilled or deionized water. It should be noted that mysids are sensitive to nitrite, and will not survive or reproduce in water containing more than 0.05 mg nitrite/L. The water can be checked for this ion by using an inexpensive (color comparison) test kit that is available at most aquarium supply stores.

The culture media should be aged to allow the build-up of bacteria in the substrate. To expedite the aging process, add 5 mL of a concentrated suspension of 48-h old *Artemia* daily. After seven days, the suitability of the new medium is checked by adding 20 mysids. If the organisms survive for 96 h, the culture medium is ready for stocking.

4. Culture Conditions

Maintain the temperature within a range of 24°C to 26°C for reproduction. Provide 12 to 16 h illumination daily at 50 to 100 ft c.

Maintain dissolved oxygen above 60% saturation by vigorous aeration with an air stone.

5. Feeding

Frequent feeding of live food is necessary to prevent cannibalism of the young by the adults. Newly hatched brine shrimp (Artemia) are fed to mysids twice daily. Tropical fish food flakes (such as Tetra-Min^R flakes) may also be used to supplement the brine shrimp diet.

6. Preparation of Brine Shrimp (Artemia)

For methods of preparing brine shrimp, see Appendix A.5.

7. Culture Maintenance

To avoid excessive build up of algal growths, periodically scrape the walls of the aquaria and standpipes, and turn over the shell substrate to cover the existing algae. In the static culture system, the mysid population tends to build to a peak and then undergoes a dramatic die off (crash) every several months. Population crashes can be prevented by removing 10% of the population every two weeks, or by transferring the brood stock to new medium in clean tanks every two months. If a decline in the brood population is observed before the scheduled date of transfer to new media, the stock should be transferred as soon as possible.

8. Production Level

At least four aquaria should be maintained to ensure a sufficient number of organisms on a continuing basis. If each aquarium is initially stocked with 200 adults, they will provide several thousand test organisms per month.

TEST ORGANISMS

Juvenile Mysidopsis almyra, M. bahia, M. bigelowi, or Neomysis americana, one to five days old, are used in the tests. To obtain the necessary number of young for a test, remove about 200 adult females (bearing embryos in their brood pouch) from the stock cultures (one adult female for each juvenile required), and place them in a large (10 cm X 15 cm), standard, fish transfer net that is partially submerged in an 8-L aquarium containing 4 L of culture medium removed from a brood stock tank. As they are released from the brood pouches, the juveniles will drop through the fish net into the aquarium, thus preventing cannibalism by the adults. The adults and juveniles in the aquarium must be fed twice daily with Artemia. The adults are allowed to remain in the net for 48 h, and are then returned to the stock tanks. The juveniles that are produced in the small tank may be used in toxicity tests over a five-day period.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

3. FATHEAD MINNOW (PIMEPHALES PROMELAS)

(Prepared by Donald J. Klemm)

DISTRIBUTION

The fathead minnow is widely distributed in North America (Fig. 19). It is a popular bait fish, and the ease with which it is propagated has led to its widespread introduction both within and outside the native range of the species. The species has been so widely distributed in the eastern and southwestern United States by bait transportation that it is difficult to determine its original range. The presumed native distribution (Vandermeer, 1966; Scott and Crossman, 1973; Lee, *et al.* 1980) extended from the Great Slave Lake in the northwest to New Brunswick, in eastern Canada, southward throughout the Mississippi valley in the United States, to southern Chihuahua in Mexico. Distribution records for this species also now include Oregon (Andreasen, 1975), and the Central Valley (Kimsey and Fisk, 1964) and other locations in California (Andreasen, 1975), but there are no records for British Columbia.

The fathead minnow is found in a wide range of habitats. This species is most abundant in brooks, small streams, creeks, ponds, and small lakes. Trautman (1957) and Scott and Crossman (1973) reported that species associated with the fathead minnow seem to vary greatly throughout its range. The fathead minnow is uncommon or absent in streams of moderate and high gradients and in most of the larger and deeper impoundments. It is tolerant of high temperature and turbidity, and low oxygen concentrations.

The fathead minnow is primarily omnivorous although Coyle (1930) reported finding algae to be one of its main foods in Ohio. Elsewhere in the United States young fish have been reported to feed on organic detritus from bottom deposits and unicellular and filamentous algae and planktonic organisms. Adults feed on aquatic insects, worms, small crustaceans, and other animals. Scott and Crossman (1973) and others regard the fathead minnow as a highly desirable forage fish, providing food for other fishes and birds.

LIFE CYCLE

The natural history and spawning behavior (Markus, 1934; Flickinger, 1973; Andrews and Flickinger, 1974; and others) of the fathead minnow are well known because of the early interest in raising the fish for bait and for feeding other pond fish, such as black bass. Male and female fathead

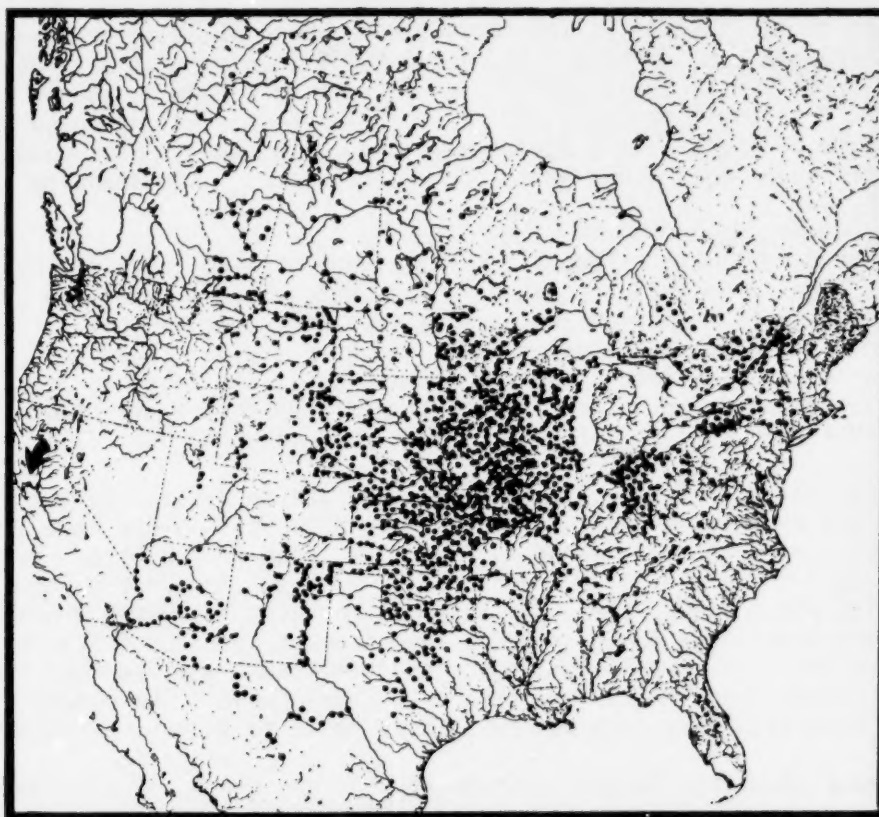


Figure 19. Map showing the distribution of the fathead minnow in North America. Open circles represent transplanted populations. Most Atlantic slope records are probably transplanted populations. (From Lee *et al.*, 1980).

minnows show sexual dimorphism at maturity. The breeding males develop a conspicuous, narrow, elongated, gray, fleshy pad of spongy tubercles on the back, anterior to the dorsal fin, and two or three rows of strong nuptial tubercles across the snout. The sides of the body of breeding males becomes almost black except for two wide vertical bars which are light in color. The females remain quite drab.

In the wild, adult fathead minnows spawn in the spring when the water temperature reaches 16° C to 18° C, and they continue to spawn throughout most of the summer. Carlander (1969) stated that the minimum temperature for spawning is 16°C.

Markus (1934) reported that fathead minnows always spawn at night, and Isaak (1961) indicated they spawned at night and sometimes during the day. Gale and Buynak (1982) and others reported that spawning often began before dawn and was usually completed before noon.

The males first select sites for spawning, such as a log or branch, rock, board, tin can, or almost any other solid inanimate object, usually in water from 7 cm to 1 m in depth. An interested female is sought out and herded into position below the nest site. After circling below the nesting site the female is nudged and lifted on the male's back until she lies on her side immediately below the undersurface of the spawning substrate.

The spawning female releases a small number of eggs (usually 100 to 150) at a time. The eggs are adhesive and attach to the underside of the spawning substrate. The females have an ovipositor (urogenital structure) to help deposit the eggs on the under side of objects. Flickinger (1966) indicated that the ovipositor is noticable at least a month prior to spawning. The reported size of the eggs varies from 1.15 mm (Markus, 1934) to 1.3 mm in diameter (Wynne-Edwards, 1932).

Immediately after the eggs are laid, they are fertilized by the male, and the female is driven off. Once eggs are deposited in the nest, the male becomes very aggressive and will use the large tubercles on his snout to help drive off all intruding small fishes. In addition to fertilizing and guarding the eggs, the male agitates the water around the eggs, which ventilates them and keeps them free of detritus. Some males will spawn with a number of females on the same substrate, so that the nest may contain eggs in various stages of development. The number of eggs per nest will vary from as few as nine or 10 to as many as 12,000.

The ovaries of the females contain eggs in all stages of development, and they spawn repeatedly as the eggs mature. A female may deposit eggs in more than one nest. Although the average number of eggs per spawn per female is generally 100 to 150, large females may lay 400 to 500 eggs per spawn.

Gale and Buynak (1982), in a study using five captive pairs of fathead minnows in separate outdoor pools, stated that each pair produced 16 to 26 clutches of eggs between May and August. The time between spawns, which ranged from two to 16 days, was affected by water temperature. As temperature increased, the intervals between spawning sessions become shorter and more uniform. In their study, from nine to 1,136 (mean of 414) eggs were spawned per clutch. The average number of eggs spawned per session by individual females ranged from 371 to 480, and the total number of eggs spawned per female ranged from 6,803 to 10,164 (mean of 8,604). The length of the spawning period during a given season also varied greatly with individual females. They suggested that the fecundity of fathead minnows is much higher than has generally been recognized, but they noted that fecundity of fish in the natural environment, where conditions might be more or less favorable, might differ from that of captive fish.

The hatching time depends on temperature. The average hatching time required at 25° C is 4.5 to 6 days. The newly hatched young (fry) are about 5 mm long, white in color, with large black eyes. In warm, food-rich water, growth is rapid. Markus (1934) stated that fish hatched

in May in Iowa reached adult size and were spawning by late July. Hubbs and Cooper (1935) and others noted that such rapid growth is unlikely in more northerly waters, and that the young do not spawn the first year. In cooler water the adult size is probably not reached until the second year. The males generally grow faster than the females, a characteristic of minnow species.

The fathead minnow is short lived, and rarely survives to the third year. However, Scott and Crossman (1973) stated that the age has been found to vary throughout the geographic range of the species. Several authors indicate that postspawning mortality in fathead minnows is often great, but Gale and Buynak (1982) stated that postspawning mortality did not occur in their study. However, they indicated that in nature, where males have to fight off intruders to defend their territory, and where they may become weakened by a lack of food over a prolonged period, their resistance to disease may be lowered. Also, at this time many waters are warm and somewhat stagnate, and conditions favor the spread of parasites and disease.

TAXONOMY

The specific name (Pimephales promelas) appears to be incorrectly applied to this fish because the fathead minnow does not fit the description originally given by Rafinesque (1820) (Lee et al., 1980). Common names include "northern fathead minnow", and "blackhead minnow" in addition to fathead minnow. The holotype was collected near Lexington, Kentucky.

Some geographic variations have been noted in the morphology of the fathead minnow. Vandermeer (1966) indicated that the introduction of this species outside its native range may have resulted in some local deviations from broad patterns of geographic variation in taxonomic characters. Some populations have been designated as subspecifically distinct: Pimephales promelas promelas, the northern form; P. p. harveyensis, the Harvey Lake form, from Isle Royal in Lake Superior and P. p. confertus, the southern form (Hubbs and Lagler, 1949, 1964). However, Taylor (1954), Vandermeer (1966), and others expressed doubt concerning the validity of assigning subspecific status to the variants and recommended against their recognition. Vandermeer (1966), in a statistical analysis of the geographic variations taxonomic characters, stated that two of the three described subspecies intergrade clinally. Of the eight characters measured, two showed a north-south trend; (1) eye diameter, with the northern fish having smaller eyes, and (2) completeness of the lateral line, with the northern fish having the least complete lateral line. However, Scott and Crossman (1973), indicated that some Canadian populations exhibit a nearly complete lateral line. The American Fisheries Society (1980), recognizes only one species.

1. Morphology and Identification (General characters)

Fathead minnows vary greatly in many characteristics throughout their wide geographic range. The morphology and characters for identification

are taken from Trautman (1957), Clay (1962), Hubbs and Lagler (1964), Eddy and Hodson (1961), and Scott and Crossman (1973). They are small fish, being typically 43 mm to 102 mm, or an average about 50 mm, in total length (Fig. 20). The first rudimentary ray of the dorsal fin is more or less thickened and distinctly separated from the first well-developed ray by a membrane. The lateral line is usually incomplete, but may be complete in specimens from some geographic areas. The scales are cycloid and moderate in size. Andrews (1970), reporting on fish collected in Colorado, noted that no scales were found on fish smaller than 14 mm, and the average length for first scale formation was 16.3 mm. The scales in the lateral series number 41 to 54.

The mouth is terminal. The snout does not extend beyond the upper lip and is decidedly oblique. Nuptial tubercles occur on mature males only, are large and well-developed on the snout, and rarely extend beyond the nostrils. They occur in three main rows, with a few on the lower jaw. In addition to nuptial tubercles, there is an elongate fleshy or spongy pad extending in a narrow band from the nape to the dorsal fin. Wide anteriorly, the pad narrows to engulf the first dorsal ray. A dark spot is usually present in front of the dorsal fin in mature males.

The peritoneum is brownish-black, and the intestine is long and coiled one or more times. A dusky band is usually absent on the snout and opercles except in some young; a lateral band across the body is distinct only in some young and in adults from clear and weedy waters. The band may be absent in breeding males, or if present, it becomes very diffuse anteriorly. This band is usually most apparent on preserved specimens. Dymond (1926), Trautman (1957), and others described the saddle-like pattern often associated with breeding males in which a light area develops just behind the head and another beneath the dorsal fin, the areas between producing a saddle affect. A narrow, dark, vertical bar or spot is present at the base of the caudal fin, but often is not very distinct. The standard lengths are usually less than four and one-half times the body depth.

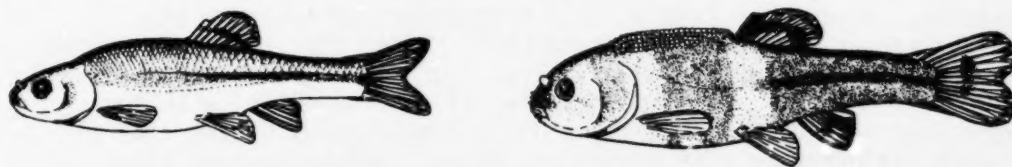


Figure 20. Fathead minnow: adult female (left) and breeding male (right). (From Eddy and Hodson, 1961).

2. Hybridization:

Trautman (1957) stated that under some conditions the fathead minnow may hybridize with the bluntnose minnow, Pimephales notatus (Rafinesque). He also indicated that fathead and bluntnose minnows were competitors, and that the fathead occurred in greater population densities only where the bluntnose was absent or comparatively few in number.

CULTURE METHODS

1. Sources of Organisms

When test fish are secured from an outside source, there is no guarantee that they will be of the age, size, quality or condition needed for performing bioassays. Additionally, because of the possibility of occurrence of disease in fish brought into the laboratory, it may be necessary to give them a prophylactic treatment for disease to prevent and/or eliminate infections. Such treatment places a stress on the test fish and requires a quarantine period of seven days.

To avoid the problems associated with using fish of unknown condition and age, an inhouse laboratory culture facility can be developed to provide a continuous supply of eggs (embryos) and/or other developmental stages of known age, which are free of disease or other stress, for use in toxicity tests.

2. Laboratory Culture Facility

Fathead minnows can be cultured in either a static or flow-through system. Flow-through systems require large volumes of water and may not be feasible in some laboratories. The culture facility consists of the following components:

- o Water supply.
- o Spawning tanks in which eggs are produced by sexually mature adults.
- o Holding tanks in which replacement spawners are held.
- o Egg incubation units in which fertilized eggs are placed to hatch.
- o Rearing tanks in which the hatched young fish are held until used in toxicity tests.

a. Water Supply

(1) Water Quality

Synthetic water or dechlorinated tap water can be used, but untreated well water is preferred. If a static system is used, it is necessary to equip each aquarium with a carbon filter system (similar to those sold for tropical fish hobbyists) to control the accumulation of metabolic wastes in the water.

(2) Dissolved oxygen

The dissolved oxygen concentration in the culture tanks should be maintained near the 100% saturation level, using air stones if necessary. Brungs (1971) in a carefully controlled long-term study, found that the growth of fathead minnows was reduced significantly at all dissolved oxygen concentrations below 7.9 mg/L. Soderberg (1982) presented an analytical approach to the reaeration of flowing water for culture systems.

(3) Maintenance

Adequate procedures for culture maintenance must be followed consistently to avoid poor water quality of the culture system. Tanks are cleaned monthly or more often as required. They should be kept free of debris (excess food, detritus, waste, etc.) by siphoning the accumulated materials (such as dead brine shrimp nauplii or cysts) from the bottom of the tanks daily with a pipette. To avoid excessive build-up of algal growth, periodically scrape the wall of aquaria. Activated charcoal and filter pads in the aquaria filtration systems should be changed weekly. Culture water may be maintained by preparation of synthetic water or use of dechlorinated tap water. Distilled or deionized water is added as needed to compensate for evaporation.

b. Spawning Tanks

The spawning unit is designed to simulate conditions in nature conducive to spawning. Fathead minnows spawn in spring and summer, and the lab photoperiod is maintained to mimic the natural spawning conditions. For breeding tanks, it is convenient to use 76 L (20 gal) aquaria. Spawning tanks must be held at a temperature of $25 \pm 2^\circ\text{C}$. Each aquarium is equipped with a heater, continuous filtering unit, and spawning substrates. The photoperiod for the spawning tanks must be rigidly controlled and maintained at 16 h light and 8 h dark (5:00 AM to 9:00 PM is a convenient photoperiod). An illumination level of 50 to 100 ft c is adequate. The breeding fish are fed all the frozen brine shrimp and tropical fish flake food or dry commercial fish food that they can eat twice daily (8:00 AM and 4:00 PM) during the week and once a day on weekends. If properly maintained, each breeding tank will produce a spawn of 100 to 200 eggs approximately every four days.

In nature, the female deposits her adhesive eggs on the underside of sticks, rocks, or boards, and the male then fertilizes, guards, and cleans the eggs. It is necessary, therefore, to provide a surface for the laying and fertilizing of eggs, as well as a territory for the male. The number of substrates placed in each tank depends on the size of the tank and the number of males. Two substrates are used for each male. The substrates should be placed equi-distant from each other and staggered from each other on the bottom of the tanks so that the spawning territories of the males do not overlap.

The recommended spawning substrates consist of inverted 15-cm (6-in.) sections of 10-cm (4-in.) diameter clay tile or PVC cut in half longitudinally. Four substrates are used in each 76-L (20 gal) spawning tank.

The number of fish in each aquarium, as well as the ratio of females to males, depends on the size of the aquarium. Five to eight females and two males are placed in each breeding tank.

Sexing of the fish to ensure a correct female/male ratio in each tank can be a problem. However, the task usually becomes easier as experience is gained. Sexually mature females usually have large bellies and a tapered snout. The sexually mature males are usually distinguished by their larger overall size, dark vertical color bands, and the spongy nuptial tubercles on the snout. Unless the males exhibit these secondary breeding characteristics, no reliable method has been found to distinguish them from females. However, using the coloration of the males and the presence of an enlarged urogenital structures and other characteristics of the females, the correct selection of the sexes can usually be achieved by trial and error.

If by accident, more than two males are placed in a tank, the less aggressive males will not mature, and may continue to be confused with females. Sexually immature males are usually recognized by their aggressive behavior and partial banding. These undeveloped males must be removed because they will eat the eggs and constantly harass the mature males, tiring them and reducing the fecundity of the breeding unit. Therefore, the fish in the spawning tanks must be carefully checked periodically for extra males.

The female contains eggs in all stages of development, and spawns repeatedly as the eggs mature until she has spawned her entire egg complement. Therefore, spawning females must be replaced periodically (every two to three months) with other mature, egg-laden females to ensure a continuous supply of eggs.

c. Replacement Spawners

Young males removed from spawning tanks and other surplus mature males are placed in all-male holding tanks for future use as spawners. Similarly, young and surplus mature females are held in

all-female holding tanks until needed as spawners. Tanks holding replacement spawners need not be temperature-controlled, but for ease of transfer to the spawning tanks, temperatures close to those of the spawning tanks should be maintained.

d. Spawning, Egg Collection, and Incubation

Once spawning conditions are right, eggs are produced. Fathead minnows spawn in the early morning hours. They should not be disturbed except for a morning feeding (8:00 AM) and daily examination of substrates for eggs between 9:30 AM and 10:30 AM. In nature, the male protects, cleans, and aerates the eggs until they hatch. In the laboratory, however, it is necessary to remove the eggs from the tanks to prevent them from being eaten by the adults, and for ease of handling for purposes of recording egg count and hatchability, and use in embryo-larval and larval tests.

The substrates are each lifted carefully and checked for newly spawned eggs. If eggs are observed on a tile, remove it immediately from the spawning tank, stand on end in a small (1- to 2-L) tank, and place an active air stone at the bottom of the tank near the egg mass. Vigorous aeration inhibits fungal growth better than weak aeration.

Alternately, the eggs can be removed from the substrate with a razor blade or with a rolling action of the index finger, placed in a 1-L jar or beaker, and aerated with sufficient vigor to keep them in suspension.

If fungal growth is a problem, it can be controlled for the entire incubation period by adding 3 mL of 1% methylene blue stock solution per liter of water in the incubation vessels (see Herwig, 1979, pp. 160-161).

During the incubation period, the eggs are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs, and eggs that have become infected by fungus, should be removed with forceps using a table top magnifier illuminator. Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Removal of fungus should be done quickly, and the substrates should be returned to the incubation tanks as rapidly as possible so that the good eggs are not damaged by desiccation.

Hatching takes four to five days at an optimal temperature of 25°C. Hatching can be delayed several (two to four) days by incubating at 10 to 15°C.

e. Rearing Tanks

(1) Transfer and feeding of newly-hatched fry.

Newly-hatched fish are transferred daily from the egg incubation tanks to small (8-L) rearing tanks, using a large bore pipette, until the hatch is complete. New rearing tanks are set up on a weekly basis to separate fish by age group. A density of 150 fry per liter is suitable for the first four weeks. The rearing tanks are allowed to follow ambient laboratory temperatures of 20 to 23°C, but sudden, extreme variations in temperature must be avoided.

Fathead minnow fry are fed freshly-hatched brine shrimp (*Artemia*) nauplii twice daily until they are four weeks old. Utilization of older (larger) brine shrimp nauplii will result in starvation of the young fish because they are unable to ingest the larger food organisms.

See Appendix A.5 for instructions on the preparation of brine shrimp nauplii.

(2) Feeding and stocking density of fish older than four weeks.

Fish older than four weeks are fed frozen brine shrimp and commercial fish starter (#1 and #2), which is ground fish meal enriched with vitamins. As the fish grow, larger pellet sizes are used, as appropriate.

3. Disease Control

Bacterial or fungal infections are the most common diseases encountered. However, if normal precautions are taken, disease outbreaks will rarely, if ever, occur. If disease occurs, treat with 0.6 mL Procaine Penicillin-G per 76-L tank (see Table 2). Hoffman and Mitchell (1980) have put together a list of some chemicals that have been used commonly for fish diseases and pests.

In aquatic culture systems where filtration is utilized, the application of certain antibacterial agents should be used with caution. A treatment with a single dose of antibacterial drugs can interrupt nitrate reduction and stop nitrification for various periods of time, resulting in changes in pH, and in ammonia, nitrite and nitrate concentrations (Collins *et al.*, 1976). These changes could cause the death of the culture organisms.

Do not transfer equipment from one tank to another without first disinfecting it. If an outbreak of disease occurs, any equipment, such as nets, airlines, tanks, etc., which has been exposed to diseased fish should be disinfected with sodium hypochlorite.

TEST ORGANISMS

Fish one to 90 days old are used in the toxicity test. Before the fish are removed, most of the water in the holding tank containing the fish should be siphoned off. The fish are then transferred to finger bowls, using a large-bore, fire-polished glass tube (6 mm to 9 mm I.D. X 30 cm long) equipped with a rubber bulb. It is important to note that larvae should not be handled with a dip net. Dipping small fish such as these with a net will result in very high mortality. The same large-bore, fire-polished glass tube discussed above should be used to transfer the fish from the finger bowl to the test vessels. The fish are counted as they are caught and are placed gently into the test vessels.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

4. SILVERSIDES (MENIDIA)

(Prepared by Douglas Middaugh)

DISTRIBUTION

Silversides occur in estuaries along the Atlantic and Gulf coasts. The Atlantic silverside, Menidia menidia, is a resident of estuaries from Maine to northern Florida. It occurs at intermediate to high salinities, typically of 12 to 30 parts per thousand (ppt), and remains in Atlantic estuaries throughout most of the year (De Sylva et al., 1962; Dahlberg, 1972). Recent evidence indicates an offshore migration at northern latitudes in the fall and reappearance of adults in estuaries in late spring (Conover and Kynard, 1981). This species is an important component in estuarine ecosystems, serving as forage fish for commercially and recreationally valued species such as striped bass, bluefish and spotted seatrout (Merriman, 1941; Bayliff, 1950; Middaugh, 1981).

Although the culturing methods described in this section were written primarily for Menidia menidia, they are also suitable for the inland silverside, M. beryllina, and the tidewater silverside, M. peninsulae. The staff of the Environmental Research Laboratory, Gulf Breeze, Florida, is currently developing procedures for spawning, culturing, and testing of other fishes, including the California grunion, Leuresthes tenuis, and the Pacific surf smelt, Hypomesus pretiosus. The availability of these fishes as test organisms will permit the use of indigenous fish in toxicity tests of wastes discharged along the entire coast line of the contiguous United States and Alaska.

LIFE CYCLE

The Atlantic silverside spawns during spring and summer. Spawning runs generally occur during April - June or July at northern latitudes, and March through July or August at southern latitudes (Bayliff, 1950; Hildebrand and Schroeder, 1928; Middaugh and Lempesis, 1976). Spawning occurs in the upper intertidal zone during daytime high tides (Middaugh, 1981). Eggs are deposited on a variety of substrates which provide protection from thermal stress and desiccation (Middaugh et al., 1981; Conover and Kynard, 1981). Females typically release 200 to 800 eggs, 1.0 to 1.2 mm diameter, as they spawn. Individuals may spawn up to five or six times, at two week intervals, during the reproductive season. The life span is generally 12 to 15 months, although year class-2 fish are occasionally found (Beck, 1979).

MORPHOLOGY AND TAXONOMY

Adult Atlantic silversides attain a total length of up to 117 mm (Fig. 21A). Females in general are slightly larger than males. The first dorsal fin has three to seven, usually four or five spines. The second dorsal fin has one spine and eight or nine rays; the anal fin has one spine and 19 to 29, usually 21 to 26, rays; and the pectoral fin has 12 to 16, usually 14 or 15, rays (Robbins, 1969). Atlantic silverside embryos are easily distinguished from those of the closely related inland silverside, *Menidia beryllina*. The former have a bundle of elastic filaments attached to the chorion at one small area of insertion (Fig. 21B, C). These filaments, typically longer than the diameter of the egg, are all the same diameter. In contrast, inland silverside eggs possess one or two thick, elongated filaments, up to 50 mm long and four to nine shorter, thinner filaments (Fig. 21D, E).

CULTURING METHODS

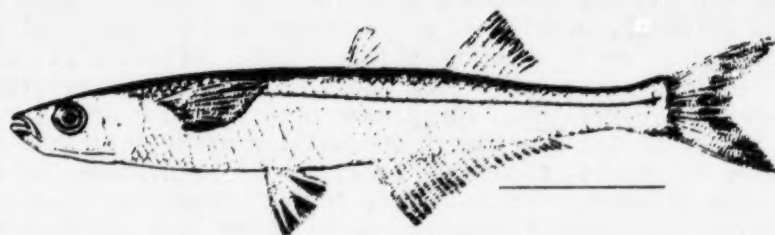
1. Sources of Organisms

a. Field Collections

The optimal time for collecting ripe *M. menidia* is just prior to daytime high tides between 8:00 AM and noon (usually one to four days after the occurrence of a new or full moon), when prespawning schools move into the upper intertidal zone (Middaugh, 1981; Middaugh *et al.*, 1981). Since the Atlantic silverside prefers relatively high salinities, it is recommended that collections be made in areas with salinities of 20 ppt or greater. Sandy beaches, bordering open but protected estuarine bays, are suitable for collecting adults. A 1 X 10-m bag seine with knotless 5-mm mesh is ideal for collecting. Since Atlantic silversides typically reside in shallow water, 1.5 m deep, they are easily captured by seining close to shore. It is important to avoid total beaching of the bag seine when collecting *M. menidia*. These fragile fish will quickly die if removed from water and, more importantly, ripe females often abort their eggs if stranded. Ideally, the bag portion of the seine, containing captured adults, should remain in water 5 to 15 cm deep (Middaugh and Lempeis, 1976).

It is possible to transport the spawn (fertilized eggs) or adults to the laboratory. The following procedure is recommended for stripping, fertilizing and transporting eggs from the field to the laboratory:

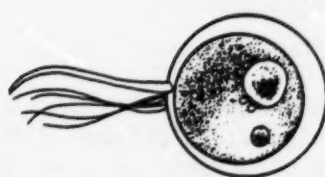
- (1) Immediately after seining (while still on the beach) three to five ripe females should be dipped into a bucket of seawater to remove sand and detritus.
- (2) Eggs are stripped into a glass culture dish containing seawater or onto a nylon screen (0.45 to 1.0 mm mesh) (Fig. 22), which is then gently lowered into a culture dish of seawater with the eggs on the upper surface of the screen (Barkman and Beck, 1976). If



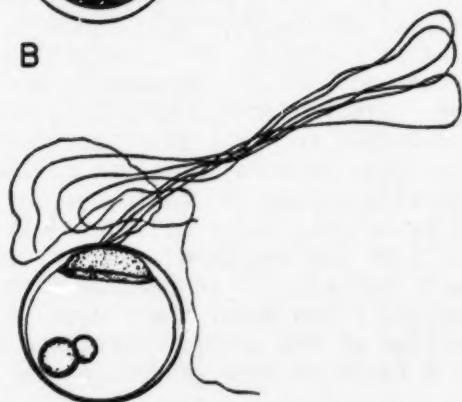
A



B



D



C



E

Figure 21. Silverside (*Menidia*): (A-C) *M. menidia*, (Atlantic silverside); (A) adult, (B) unfertilized, (C) developing embryo; note that filaments are all equal in diameter; (D-E) *M. beryllina* (inland silverside), (D) unfertilized egg, and (E) developing embryos; note one thick filament and several thin filaments.

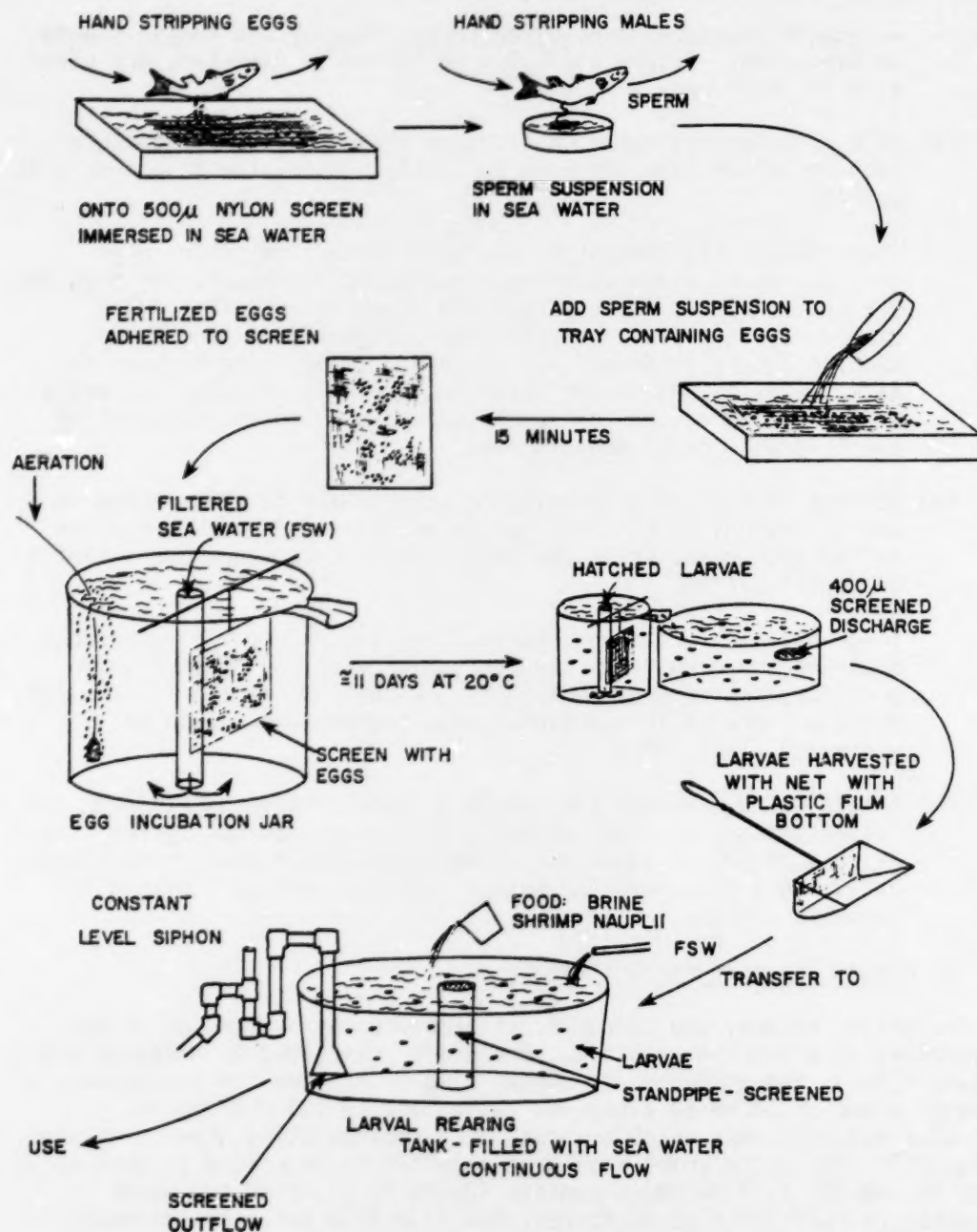


Figure 22. Techniques for collection of silverside eggs in the field and production of larvae in the laboratory (From Beck, 1979).

excessive pressure is required to strip eggs, the female should be discarded. Mature eggs, 1.0 to 1.2 mm in diameter, are clear, with an amber hue.

- (3) Milt from several males can then be stripped into the culture dish and mixed with the eggs by gently tilting the dish from side to side.

Upon contact with seawater, adhesive threads on mature eggs uncoil, making enumeration and separation difficult. If eggs are stripped directly into the culture dish, one end of a nylon string may be dipped into the dish and gently rolled so the embryos adhere (Middaugh and Lempesis, 1976). The Barkman and Beck (1976) technique for attaching the eggs to nylon screening minimizes the natural clumping tendency due to entanglement of the filaments on M. menidia eggs.

- (4) Strings of embryos or embryos on screens may be transported to the laboratory by placing them in an insulated glass container filled with seawater at the approximate temperature and salinity of fertilization.

If gravid fish are transported to the laboratory for subsequent spawning, care must be taken to avoid overcrowding of fish in transport containers. Continuous, vigorous aeration is required and any increase in container water temperature should be minimized (Beck, 1979).

A mass culture system for incubating the screen-adhered eggs and collecting the hatched larvae in a flowing seawater system (Fig. 22) was described in detail by Beck (1979). A similar procedure utilizing a recirculating system was described by Middaugh and Lempesis (1976).

b. Laboratory year-round Spawning

Atlantic, inland, and tidewater silversides may be spawned in the laboratory on a year-round basis. Procedures described by Middaugh and Takita (1983), and Middaugh and Hemmer (1984), provide for maintenance of a brood stock of 30 to 50 fish, sex ratio 1:1, in 1.3 m diameter, circular holding tanks which are part of a recirculating seawater system (Fig. 23). The photoperiod should be adjusted to 14 L:10 D (lights on at 5:00 AM and off at 7:00 PM, intensity 50-100 ft c), with the water temperature maintained at 18 to 20°C for fish from northern latitudes, and 20 to 25°C for southern latitudes. Suitable salinities for the culture units would be 25 to 30 ppt for the Atlantic and tidewater silversides, and 7 ppt for the inland silverside. Fish are fed 8 g TetraMin® each morning and afternoon, and concentrated Artemia nauplii (hatch obtained from approximately 15 mL of eggs after 48 h of incubation at 25°C) in mid-afternoon (see section on Artemia culture). Excess food should be siphoned from the holding tanks weekly. Filter media (activated charcoal) located in a reservoir tray should be changed

weekly, immediately after cleaning the holding tanks. To induce spawning by the Atlantic silverside, the circulation current velocity in the holding tanks should be reduced to zero (from 8 to 0 cm/sec) twice daily by turning off the seawater circulation pump from midnight to 1:00 AM, and from noon to 1:00 PM. Atlantic silversides will spawn in response to interrupted current velocities during daytime (noon to 1:00 PM). Spawning of the tidewater silverside also is enhanced by reducing the current velocity twice daily, but spawns primarily during nighttime. No interruption in current is necessary to enhance spawning by the inland silverside.

A suitable spawning substrate can be made by cutting enough 25 cm lengths of No. 18 nylon string to form a small bundle, and tying a string around the middle of the bundle to form a "mop." The mop is suspended just below the surface of the water, in contact with the side of the holding tanks. Spawning fish will deposit eggs on this substrate. The mops are removed from the holding tanks daily and suspended in incubation vessels. Typical egg production ranges from 300 to 1200 per spawn. Fish generally can be expected to spawn three to four days each week.

It is essential that light-tight curtains surround the holding tanks. These curtains should remain closed except during periodic feedings, tanks cleaning, and during removal and replacement of spawning substrates.

2. Culture Vessels

Embryos attached to nylon screening or nylon string may be suspended in a culture system such as shown in Fig. 22. The culture chambers for embryos should be constructed of glass. Upon hatching, larvae may be transferred from the collection container to a 90-cm diameter glass or fiberglass tank with a volume of 350 L. Tanks receive a continuous flow of seawater at 2 L/min. Water is introduced at the tank periphery causing a gentle current sufficient to induce orientation to water movement and normal schooling behavior. Water is discharged from the tank by two automatic siphons. Siphon openings are protected by a 400 μ m nylon screen to prevent escape of larvae. An inverted funnel is used at the siphon to decrease the velocity of discharge water, thus preventing impingement of larvae.

Embryos can also be incubated in small (4- to 10-L) glass aquaria, by placing the nylon screening or strings just below the surface of the water. Gentle aeration should be provided by an airstone positioned near the bottom of the holding aquaria.

3. Culture Media

Use natural seawater if it is available and unpolluted. Otherwise use synthetic seawater prepared by adding artificial marine salts, such as Instant Ocean[®] or Rila[®] Marine Mix, to distilled or deionized water. If synthetic seawater is used, it should be aged for a least one week before being utilized in culture aquaria.

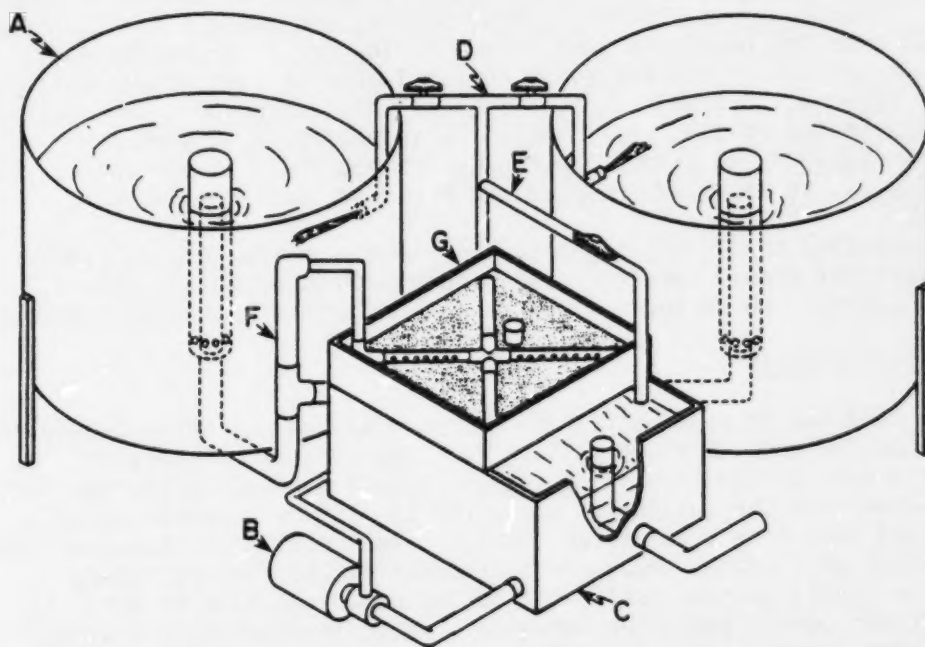


Figure 23. Holding and spawning system utilized in the culture of silversides (*Menidia*). (A) 1.3 m diameter tanks, (B) circulation pump, (C) reservoir, (D) seawater distribution system, (E) by-pass line, (F) seawater return line, and (G) reservoir filter system. (From Middaugh and Hemmer, 1984).

4. Culture Conditions

The salinity maintained during incubation should be similar to that of the water from which the adults were taken, if collected in the field, or at which the adults are being maintained in the laboratory, if the embryos originate from laboratory brood stock. Water temperature should be maintained at 20 to 25°C depending upon the latitude where fish are collected. Provide a photoperiod of 12 to 14 h of illumination daily at 50-100 ft c (12 h minimum light/24 h). Embryos will hatch in seven to 14 days, depending upon the incubation temperature and salinity (Middaugh and Lempeis, 1976).

5. Feeding and Stocking Density

Upon hatching, Menidia larvae should be fed immediately. Newly hatched brine shrimp (Artemia) nauplii (less than eight hours old) are fed larvae twice daily. It is essential to feed newly hatched Menidia larvae with newly-hatched brine shrimp. Utilization of older, larger, brine shrimp nauplii will result in starvation of the larvae since they are unable to ingest the larger food organisms. Three to four days after hatching, the fish are able to consume older (larger) brine shrimp nauplii. Methods for culturing brine shrimp are discussed in Appendix A.5. A stocking density of about 300 larvae is suitable in an 76-L aquarium.

6. Culture Maintenance

To avoid excessive build up of algal growths, periodically scrape the walls of aquaria. Activated charcoal in the aquarium filtration systems should be changed weekly and detritus (dead brine shrimp nauplii or cysts) siphoned from the bottom of holding aquaria each week. Salinity may be maintained at the proper level by addition of distilled or deionized water to compensate for evaporation.

TEST ORGANISMS

Fish one to 90 days old are used in tests. Most of the water in the holding aquarium should be siphoned off before removal of larvae. Larvae can then be siphoned from the holding tanks into a holding vessel. It is essential that larvae not be handled with a dip net, because it will result in very high mortality. A large-bore, fire-polished glass tube, 6mm I.D. x 500mm long (1/4" ID X 18" long), equipped with a rubber squeeze bulb should be used to transfer the larvae from the holding vessel to the test vessels. It is more convenient to first transfer five fish to each of several small beakers containing 20 mL of saline dilution water. The appropriate number of fish (multiples of five) can then be added to test vessels.

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APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

5. BRINE SHRIMP

SOURCES OF BRINE SHRIMP EGGS

Although there are many commercial sources of brine shrimp eggs, the Brazilian strain is preferred because it has low concentrations of chemical residues (one source is: Aquarium Products, 180 L Penrod Ct, Glen Burnie, MD, 21061). Reference Artemia cysts are available in limited quantity from the Artemia Reference Center, State University of Ghent, Belgium, J. Plafeousfruar 22, B 9000, Ghent, Belgium. In the United States, contact the Environmental Research Laboratory, USEPA, South Ferry Road, Narragansett, Rhode Island, 02882, for information on reference materials and commercial sources of good quality Artemia eggs.

INCUBATION CHAMBER AND PROCEDURE

A 2000 mL separatory funnel makes a convenient brine shrimp hatching vessel. A satisfactory but less expensive apparatus can be prepared by cutting the bottom out of a 2-L plastic soft drink bottle and inserting a rubber stopper with a flexible tube and pinch cock. Add approximately 1800 mL dechlorinated water and four tablespoons of non-iodized salt to the hatching vessel and shake until the salt dissolves (or use synthetic or filtered natural seawater). Add the desired quantity of eggs (usually 15 to 20 mL) to the vessel and mix well. The quantity of eggs used depends on feeding requirements. For example, approximately 15 mL of eggs will provide enough brine shrimp nauplii to feed three large stock cultures of mysids in 76-L aquaria, or 1000 to 1500 newly hatched fish in four to six 8-L tanks.

After the appropriate volume of eggs is added to the hatching vessel, air is vigorously bubbled through a 1-mL pipet which is lowered through the neck of the funnel so that the tip rests at the bottom. Aeration will keep the eggs and newly-hatched nauplii from settling on the bottom, where the dissolved oxygen quickly would be depleted.

The eggs will hatch in 24 h at a temperature of 27°C. Hatching time varies with incubation temperature and the geographic strain of Artemia used.

HARVESTING THE NAUPLII

The nauplii can be easily harvested in the following manner:

1. After 24 h at 27°C, remove the pipet supplying air, allow the nauplii to settle to the bottom of the separatory vessel. The empty egg shells will float to the top.

2. After approximately five minutes, using the stopcock, drain off the nauplii into a 250-ml beaker.
3. After another five minutes, again drain the nauplii into the beaker.
4. The nauplii are further concentrated by pouring the suspension into a small cylinder which has one end closed with #20 plankton netting.
5. The concentrate is resuspended in 50 mL of appropriate culture water, mixed well, and dispensed with a pipette. (Mysids require approximately 100 nauplii/mysid/day.)
6. Discard the remaining contents of the hatching vessel and wash the vessel with hot soap and water.
7. Prepare fresh salt water for each new hatch.

To have a fresh supply of Artemia nauplii daily, several hatching vessels must be set up and harvested on alternate days.

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APPENDIX B

DILUTOR SYSTEMS

Two proportional dilutor systems are illustrated: the solenoid valve system, and the vacuum siphon system.

1. Solenoid and Vacuum Siphon Dilutor Systems

The designs of the solenoid and vacuum siphon dilutor systems incorporate features from devices developed by many other Federal and state programs, and have been shown to be very versatile for on-site bioassays in mobile laboratories, as well as in fixed (central) laboratories. The Solenoid Valve system is fully controlled by solenoids (Figs. 24, 25, and 26), and is preferred over the vacuum siphon system. The Vacuum Siphon system (Figs. 24, 27, and 28), however, is acceptable. The dilution water, effluent, and pre-mixing chambers for both systems are illustrated in Figs. 29, 30, and 31. Both systems employ the same control panel (Fig. 32).

If in the range-finding test, the LC50 of the effluent falls in the concentration range, 6.25% to 100%, pre-mixing is not required. The pre-mixing chamber is bypassed by running a TYGON® tube directly from the effluent in-flow pipe to chamber E-2 (see Figures 26 and 28), and Chambers E-1 and D-1 and the pre-mixing chamber are deactivated.

The dilutor systems described here can also be used to conduct tests of the toxicity of pure compounds by equipping the control panel with an auxillary power receptacle to operate a metering pump to deliver an aliquot of the stock solution of the pure compound directly to the mixing chamber during each cycle. In this case, chamber E-1 is de-activated and chamber D-1 is calibrated to deliver a volume of 2000 mL, which is used to dilute the aliquot to the highest concentration used in the toxicity test.

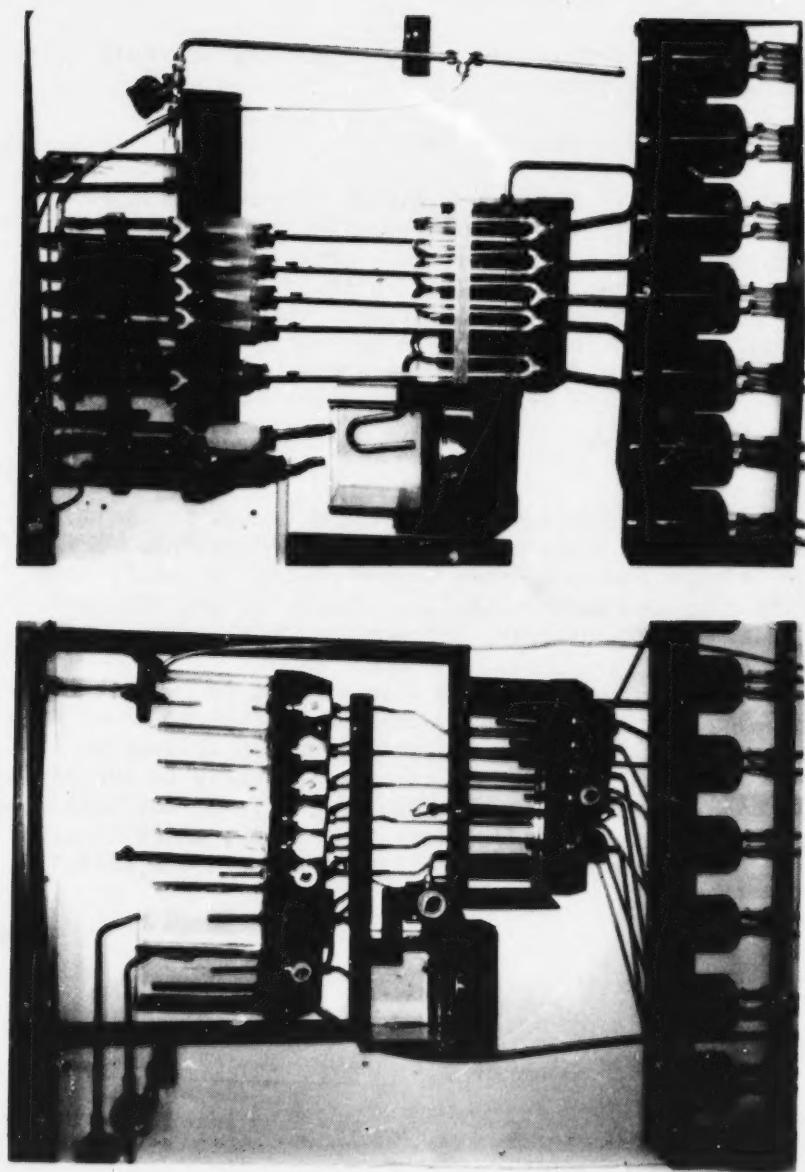


Figure 24. Photographs of the solenoid valve system (left), and the vacuum siphon system (right).

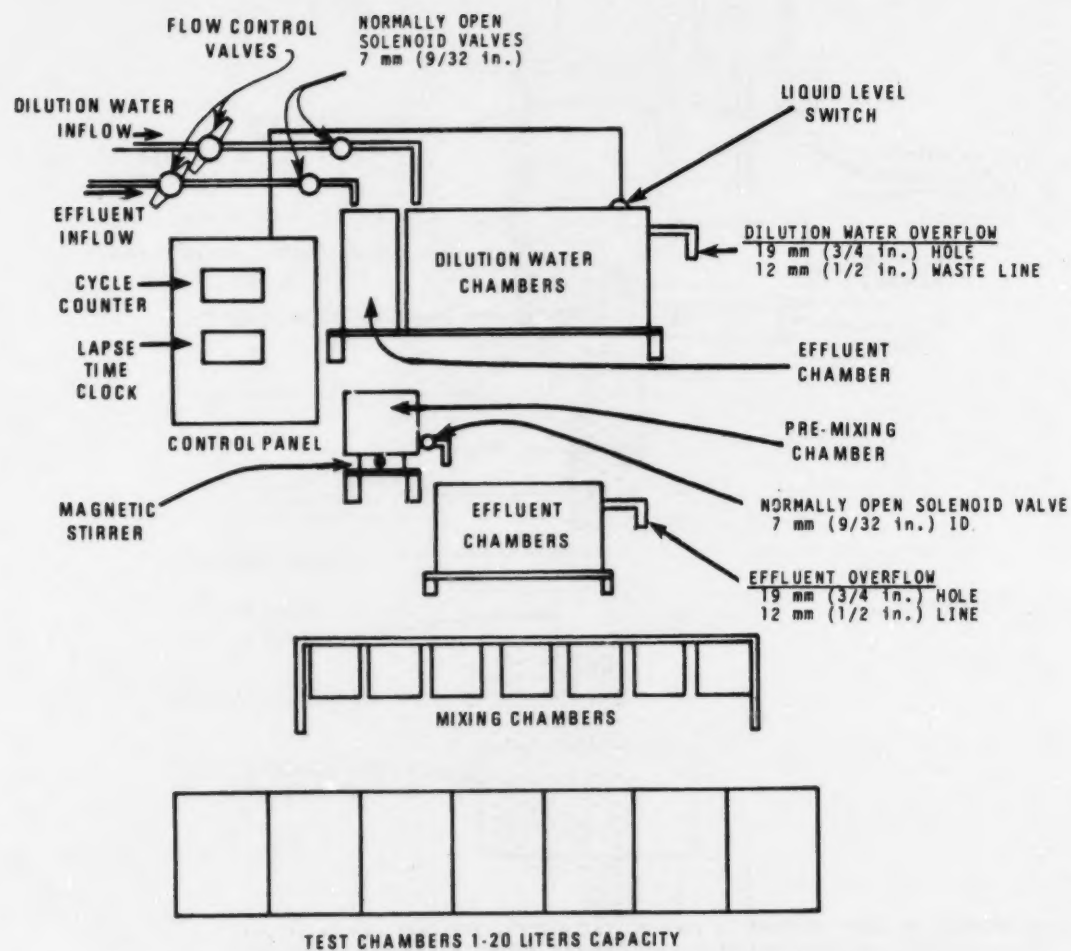
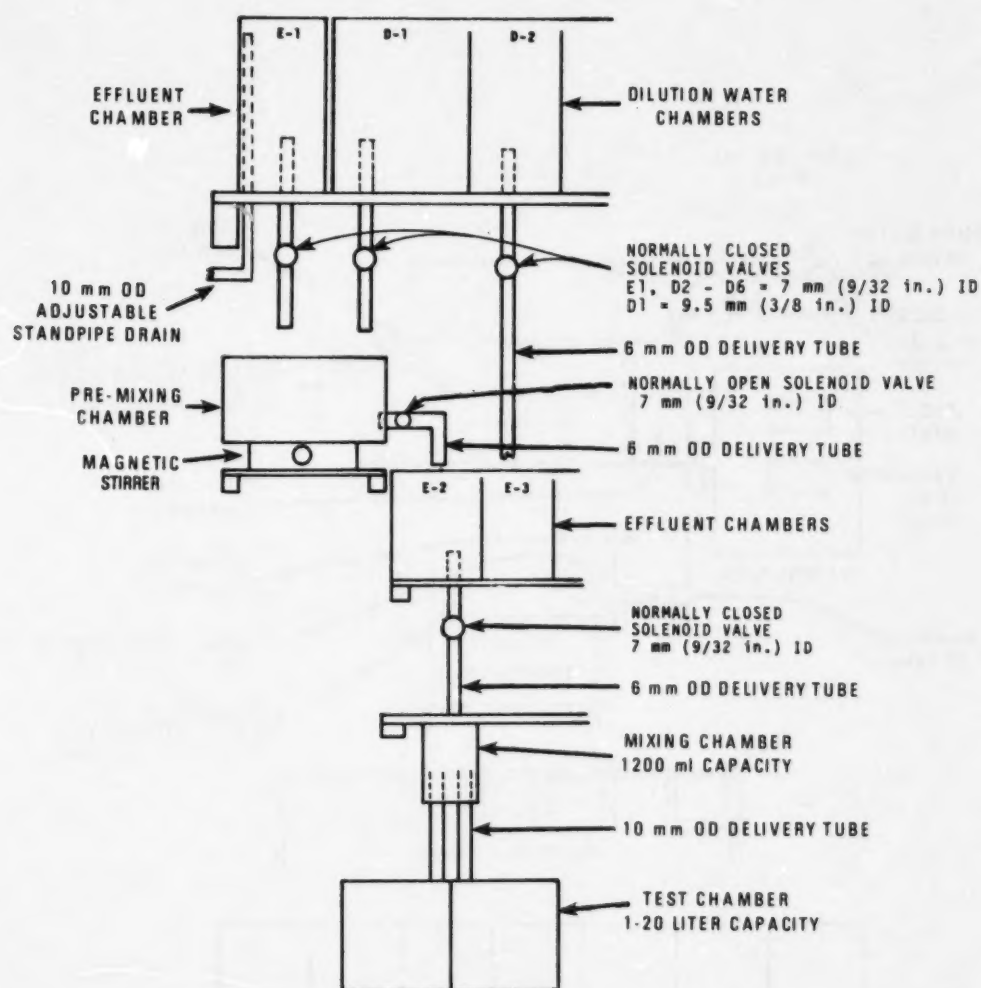


Figure 25. Solenoid valve dilutor system, general diagram (not to scale).



NOTE: WHEN 100% EFFLUENT IS USED AS THE HIGHEST EFFLUENT CONCENTRATION, E-1, D-1, AND THE PRE-MIXING CHAMBER ARE BYPASSED BY CONNECTING A TYGON TUBE TO THE EFFLUENT INFLOW, AND RUNNING IT DIRECTLY TO E-2. IN THIS CASE, SOLENOIDS FOR E-1 AND D-1, AND THE PRE-MIXING CHAMBER ARE DISCONNECTED. D-2 + E-3 = 50% EFFLUENT; D-3 + E-4 = 25% EFFLUENT, ETC.

Figure 26. Solenoid valve dilutor system, detailed diagram (not to scale).

SOLENOID SYSTEM EQUIPMENT LIST

1. Diluter Glass.
2. Stainless Steel Solenoid Valves
 - a. 3, normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes and mixing chamber pipe.
 - b. 1, normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030B65, for D-1 chamber evacuation pipe.
 - c. 12, normally closed, two-way, 36 psi, water, 1/4" pipe size, 9/32" orifice size. ASCO 8262C38, for remaining dilution chambers (D2 - D6) and effluent chamber (E1-E6) evacuation pipes.
3. Stainless steel tubing, seamless, austenetic, 304 grade for freshwater and 316 grade for saline water.
 - a. 10 ft of 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
 - b. 60 ft of 1/4" OD, 0.035" wall thickness, for dilution water and effluent pipes.
 - c. 1 ft of 3/4" OD, 0.035" wall thickness, for standpipe in D1 chamber.
4. Swagelok tube connectors, stainless steel.
 - a. 4, male tube connectors, male pipe size 1/4", tube OD 3/8."
 - b. 2, male tube connectors, male pipe size 1/2", tube OD 3/8."
 - c. 26, male tube connectors, male pipe size 1/4", tube OD 1/4."
 - d. 2, male tube connectors, male pipe size 3/8", tube OD 3/8."
 - e. 2, male adapter, tube to pipe, male size 1/2", tube OD 3/8."
5. 7, 1200 mL stainless steel beakers.
6. Several lbs each of Neoprene stoppers, sizes 00, 0, and 1; 1 lb of size 5.
7. 14 - aquarium (1-20 liters).
8. Magnetic stirrer.
9. 2 - PVC ball valves, 1/2" pipe size.
10. Diluter control panel - see Fig. 32 and equipment list.
11. Plywood sheeting, exterior grade: one - 4' x 8' x 3/4", one - 4' x 8' x 1/2".
12. Pine or redwood board, 1" x 8", 20 ft.
13. Epoxy paint, 1 gal.
14. Assorted wood screws, nails, etc.
15. 25 ft - 1/4" ID, TEFLON[®] tubing, to connect the mixing chambers to the test chambers.

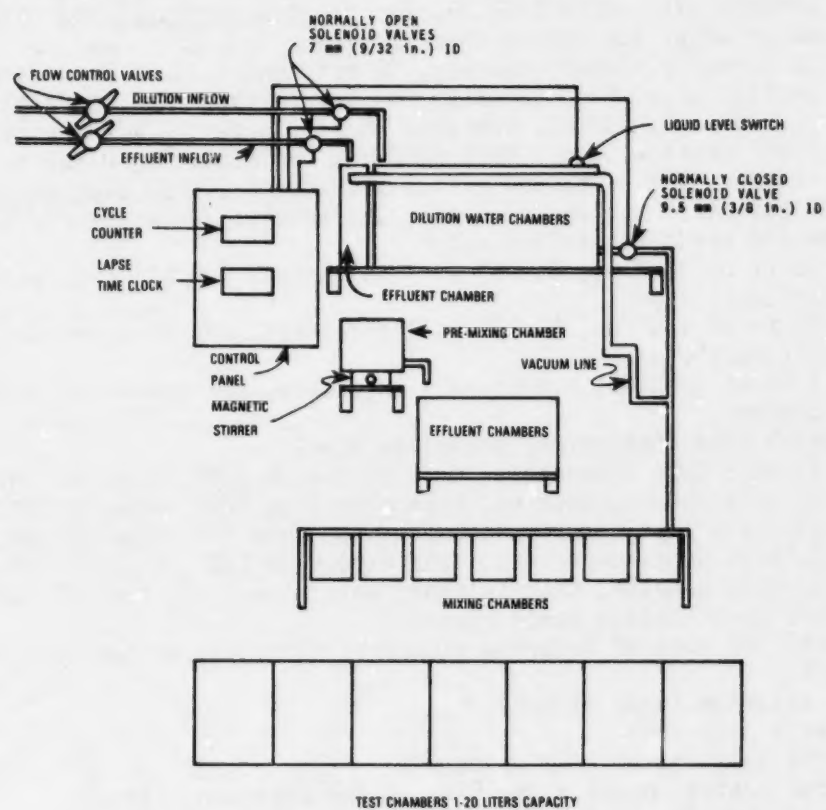


Figure 27. Vacuum siphon dilutor system, general diagram (not to scale).

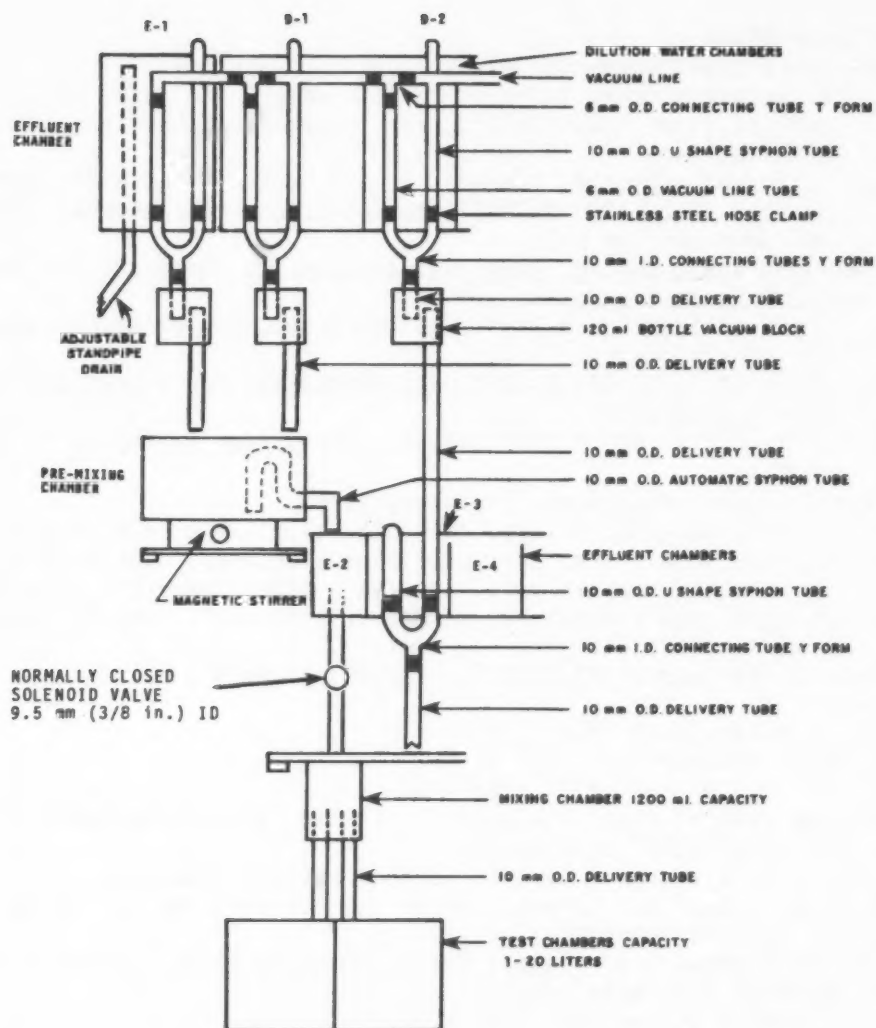
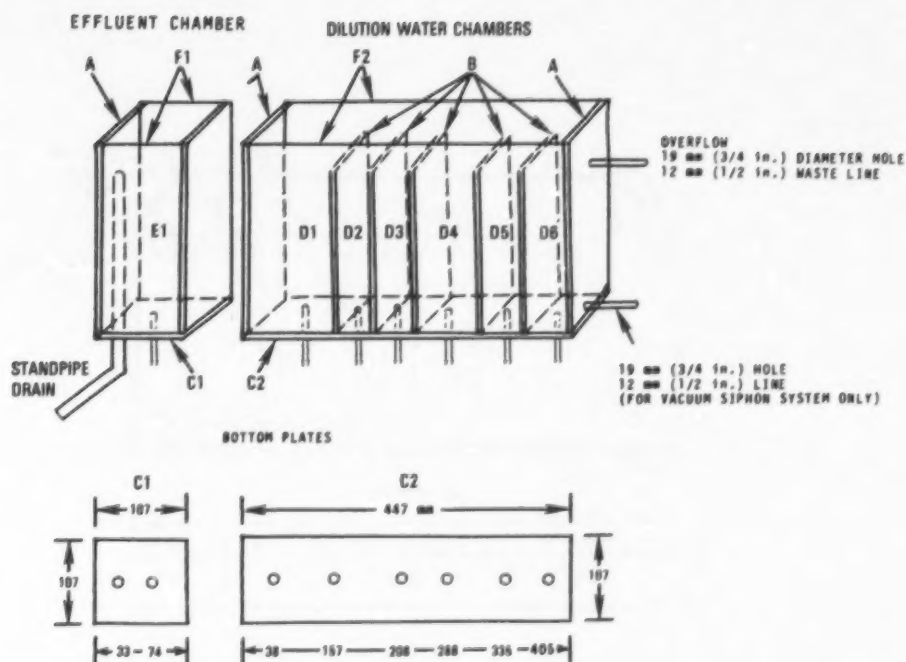


Figure 28. Vacuum siphon dilutor system, detailed diagram (not to scale).

VACUUM SIPHON SYSTEM EQUIPMENT LIST

1. Diluter Glass.
2. Stainless steel solenoid valves.
 - a. 2, normally open, two-way, 55 psi, water, 1/4" pipe size, 9/32" orifice size, ASCO 8262152, for incoming effluent and dilution water pipes.
 - b. 2, normally closed, two-way, 15 psi, water, 3/8" pipe size, 3/8" orifice size, ASCO 8030B65, for dilution water chamber D-6 and effluent chamber E-2.
3. Stainless steel tubing, seamless, austenetic, 304 grade for freshwater and 316 grade for saline water.
 - a. 60 ft of 3/8" OD, 0.035" wall thickness, for dilution water and effluent pipes.
 - b. 20 ft of 5/16" OD, 0.035" wall thickness, for standpipes in mixing chambers.
 - c. 1 ft of 3/4" OD, 0.035" wall thickness, for standpipe in D1 chamber.
4. Swagelok tube connectors, stainless steel
 - a. 4, male tube connectors, male pipe size 1/4", tube OD 3/8."
 - b. 2, male tube connectors, male pipe size 3/8", tube OD 3/8."
 - c. 2, male adapter, tube to pipe, male pipe size 1/2", tube OD 3/8."
 - d. 2, male tube connectors, male pipe size 1/2", tube OD 3/8."
5. 7, 1,200 mL stainless steel beakers.
6. Several lbs each of NEOPRENE^R stoppers, sizes 00, 0 and 1; 1 lb of size 5.
7. 14, aquarium (1-20 liters)
8. Magnetic stirrer.
9. 2, PVC Ball valves, 1/2" pipe size.
10. Diluter control panel equipment - see Fig. 32 and equipment list.
11. 7, 120 ml NALGENER^R bottles.
12. 3 ft, 1-in-2 aluminum bar, for siphon support brackets.
13. Stainless steel set screws, box of 50, for securing SS tubing in siphon support brackets.
14. Stainless steel hose clamps, box of 10, size #4 or 5,(need 3 boxes).
15. 6, NALGENER^R T's, 5/16" OD.
16. 12, TYGON^R Y connectors, 3/8" I.D.
17. TYGON^R tubing, 3/8" OD, 10 ft.
18. Plywood sheeting, exterior grade: one - 4' x 8x x 3/4", one - 4' x 8' x 1/2".
19. Pine or redwood board, 1" x 8", 20 ft.
20. Epoxy paint, 1 gal.
21. Assorted wood screws, nails, etc.
22. 25 ft of 5/16" ID, TEFLON^R tubing, to connect the mixing chambers to the test chambers.



DRAIN HOLES IN BOTTOM PLATE (C1 AND C2) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM. FOR VACUUM SIPHON DILUTOR SYSTEM, DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER E1.

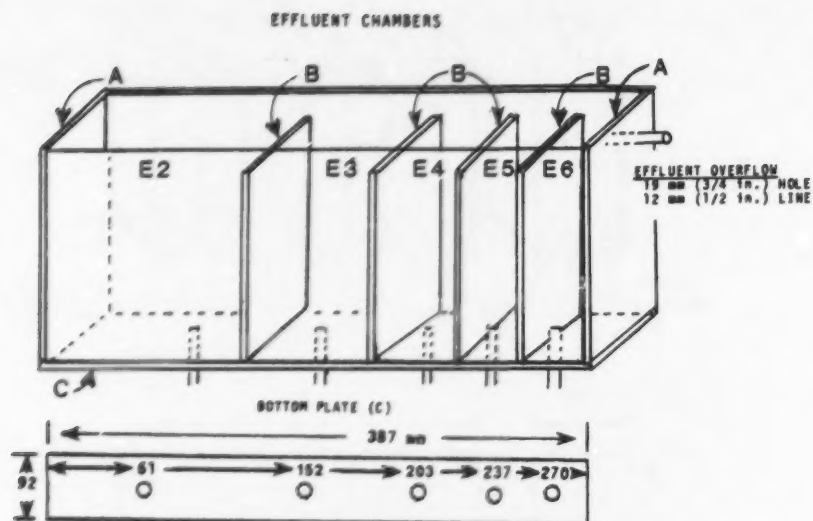
INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6 mm (1/4 in.) PLATE GLASS. NOTE: 1.6 mm (1/16 in.) No. 304 GRADE (FOR FRESH WATER) OR No. 316 GRADE (FOR SALINE WATER) STAINLESS STEEL MAY BE SUBSTITUTED FOR GLASS

LENGTH	WIDTH	NO. PIECES (16)
A: 231 mm X 95 mm	- 4 (END PLATES)	
B: 200 mm X 95 mm	- 5 (PARTITIONS)	
C1: 107 mm X 107 mm	- 1 (BOTTOM PLATE FOR E1)	
C2: 447 mm X 107 mm	- 1 (BOTTOM PLATE FOR D1-D6)	
F1: 107 mm X 231 mm	- 2 (FRONT AND BACK PANELS FOR E1)	
F2: 447 mm X 231 mm	- 2 (FRONT AND BACK PANELS FOR D1-D6)	

INSIDE CELL MEASUREMENTS AND APPROXIMATE VOLUMES

	WIDTH	LENGTH	HEIGHT	VOLUME
E1:	95 mm	X 95 mm	X 231 mm	= 2085 mL
D1:	125 mm	X 95 mm	X 200 mm	= 2375 mL
D2:	40 mm	X 95 mm	X 200 mm	= 760 mL
D3:	50 mm	X 95 mm	X 200 mm	= 950 mL
D4:	60 mm	X 95 mm	X 200 mm	= 1140 mL
D5:	60 mm	X 95 mm	X 200 mm	= 1140 mL
D6:	70 mm	X 95 mm	X 200 mm	= 1330 mL

Figure 29. Effluent and dilution water chambers (not to scale).



DRAIN HOLES IN BOTTOM PLATE (C) SHOWN FOR SOLENOID VALVE DILUTOR SYSTEM ONLY. FOR VACUUM SIPHON DILUTOR SYSTEM, A DRAIN HOLE IS REQUIRED ONLY FOR CHAMBER E2.

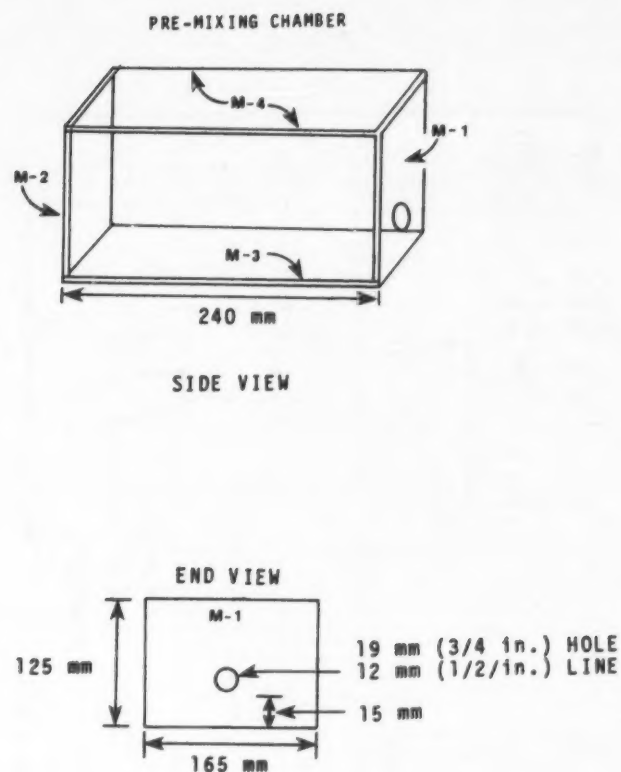
INDIVIDUAL PART SIZE AND NUMBER OF PIECES USING 6 mm (1/4 in.) PLATE GLASS ARE SHOWN BELOW. NOTE: 1/16 in. No. 304 (FOR FRESH WATER) OR No. 316 STAINLESS STEEL (FOR SALINE WATER) MAY BE SUBSTITUTED FOR GLASS.

	LENGTH	WIDTH	NO. PIECES (9)
A	180 mm X 80 mm	-	2 (END PLATES)
B	155 mm X 80 mm	-	4 (PARTITIONS)
C	296 mm X 92 mm	-	1 (BOTTOM PLATE)
D	296 mm X 180 mm	-	2 (FRONT AND BACK PLATES)

INSIDE CHAMBER MEASUREMENTS AND APPROXIMATE VOLUMES:

	WIDTH	LENGTH	HEIGHT	VOLUME
E2:	110 mm X 80 mm	X 155 mm	=	1364 mL
E3:	60 mm X 80 mm	X 155 mm	=	744 mL
E4:	30 mm X 80 mm	X 155 mm	=	372 mL
E5:	30 mm X 80 mm	X 155 mm	=	372 mL
E6:	30 mm X 80 mm	X 155 mm	=	372 mL

Figure 30. Effluent chambers (not to scale).



INDIVIDUAL PART SIZE AND NUMBER OF PIECES
USING 6 mm (1/4 in.) PLATE GLASS. APPROXIMATE
CAPACITY 4360 mL.

M-1	125 mm X 153 mm	- 1 (END PLATE, WITH HOLE)
M-2	125 mm X 153 mm	- 1 (END PLATE)
M-3	240 mm X 165 mm	- 1 (BOTTOM PLATE)
M-4	240 mm X 125 mm	- 2 (SIDE PLATES)

Figure 31. Pre-mixing chamber (not to scale).

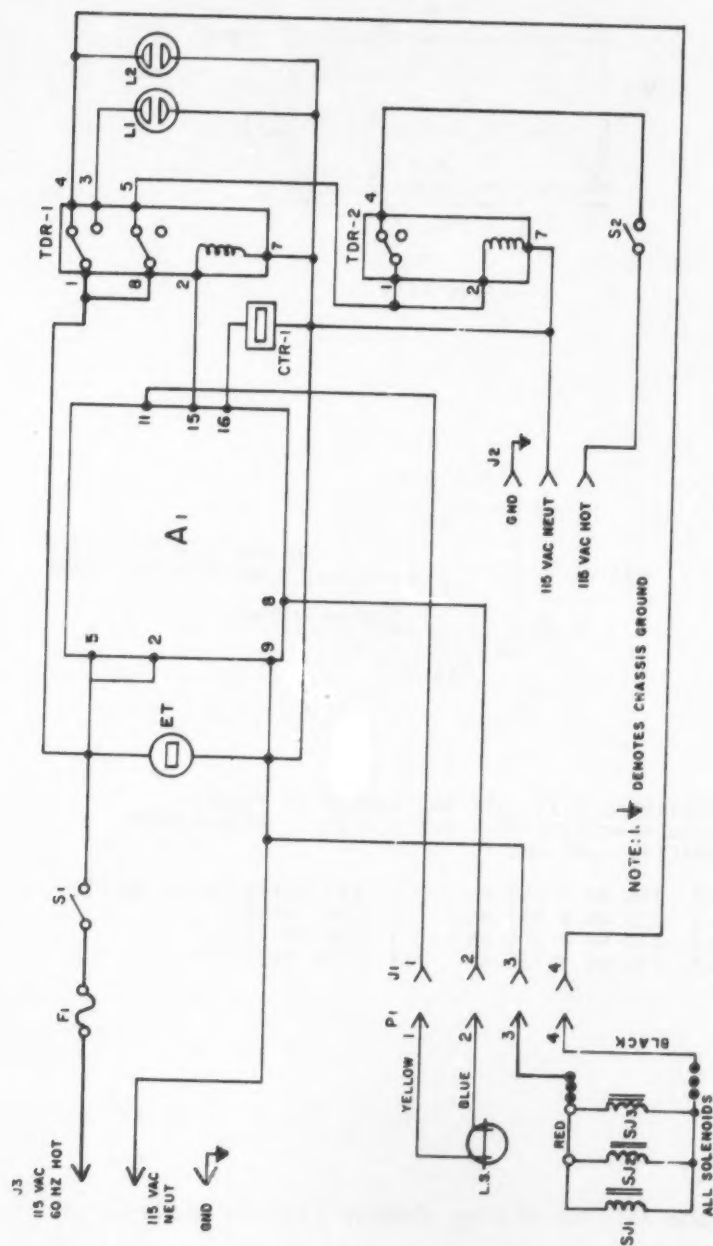


Figure 32. Dilutor control panel wiring diagram.

DILUTOR CONTROL PANEL EQUIPMENT LIST*

<u>Designation</u>	<u>CKT Description</u>	<u>Manufacturer</u>
A1	Encapsulated amplifier	Cutler Hammer 13535H98C
CTR-1	Cycle counter	Redington #P2-1006
ET	Elapsed time indicator	Conrac #636W-AA H&T
F1	Input power fuse	Little fuse 342038
J1	Recepticle	Amphenol 91PC4F
J2	Aux A.C. output jack	Stand. 3-prong AC Rcpt.
J3	Main input power cord	Stand. 3-prong AC male plug
L1	Fill indicator light	Dialco 95-0408-09-141
L2	Emptying indicator light	Dialco 95-0408-09-141
L.S.	Liquid level sensor (Dual Sensing Probe)	Cutler Hammer 13653H2
P1	Plug	Amphenol 91MC4M
S1	On-off main power switch (spst)	Cutler Hammer 7580 K7
S2	On-off aux power switch (spst)	Cutler Hammer 7580 K7
SJ1	Solenoid	(See Solenoid and Vacuum System equipment lists)
SJ2	"	" " "
SJ3	"	" " "
SJ4 -SJ16	Additional Solenoids for Solenoid Valve System	" " "
TDR-1	Time delay relay	Dayton 5x829
TDR-2	Aux time delay relay	Dayton 5x829

*Consult local electric supply house.

APPENDIX C

BIOASSAY MOBILE LABORATORY PLANS

1. TANDEM-AXLE TRAILER

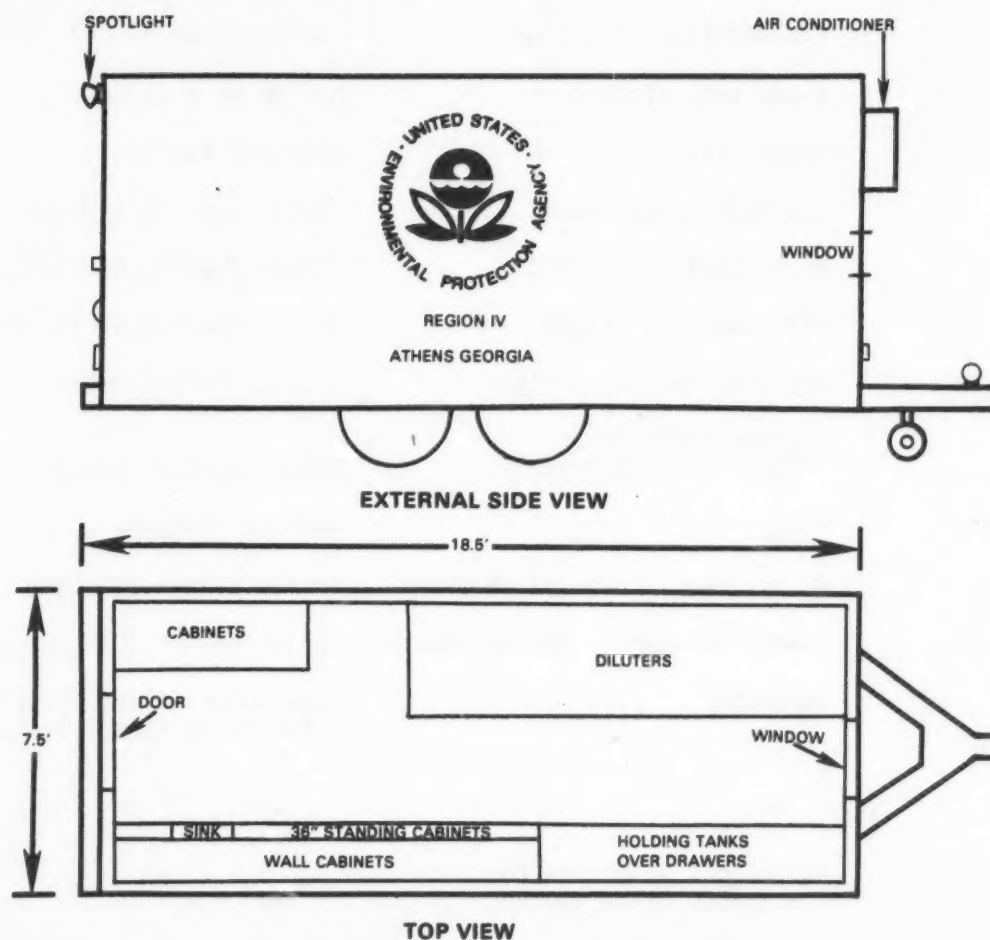


Figure 33. Mobile bioassay laboratory, tandem-axle trailer. Above - external side view; below - internal view from above.

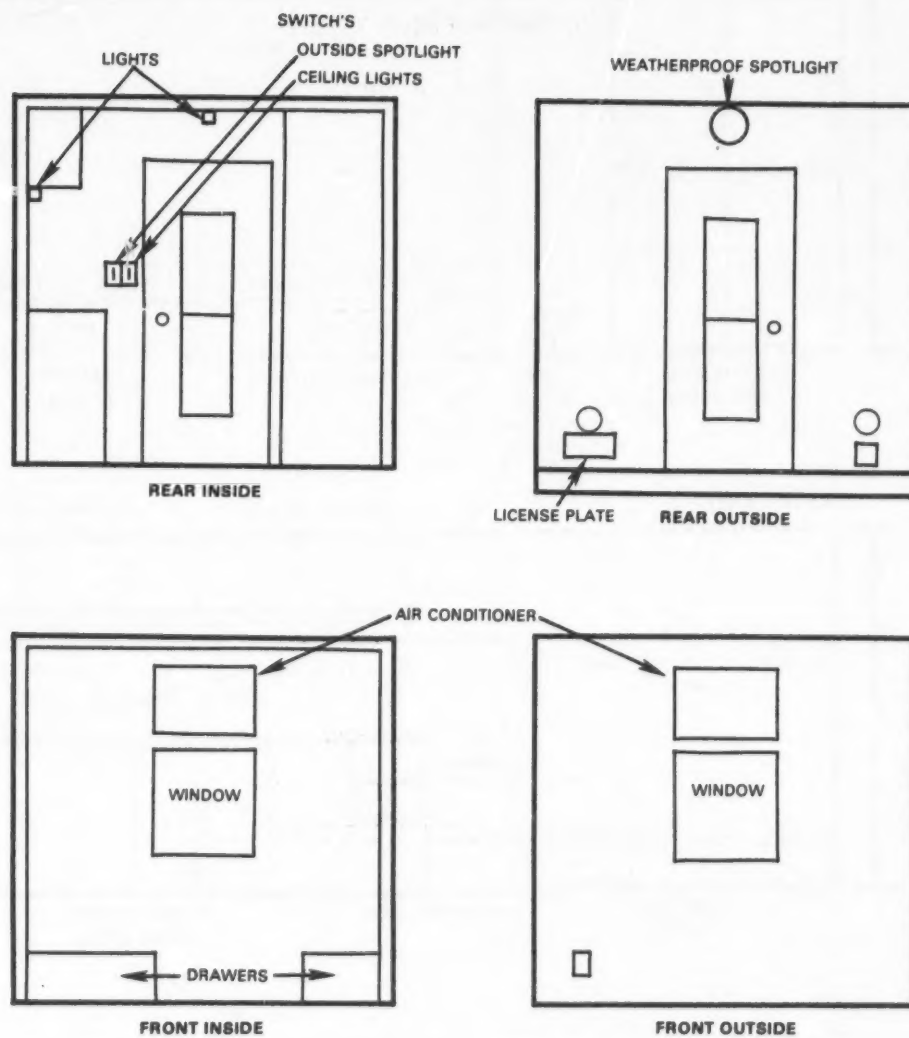


Figure 34. Mobile bioassay laboratory, tandem-axle trailer, external and internal end views.

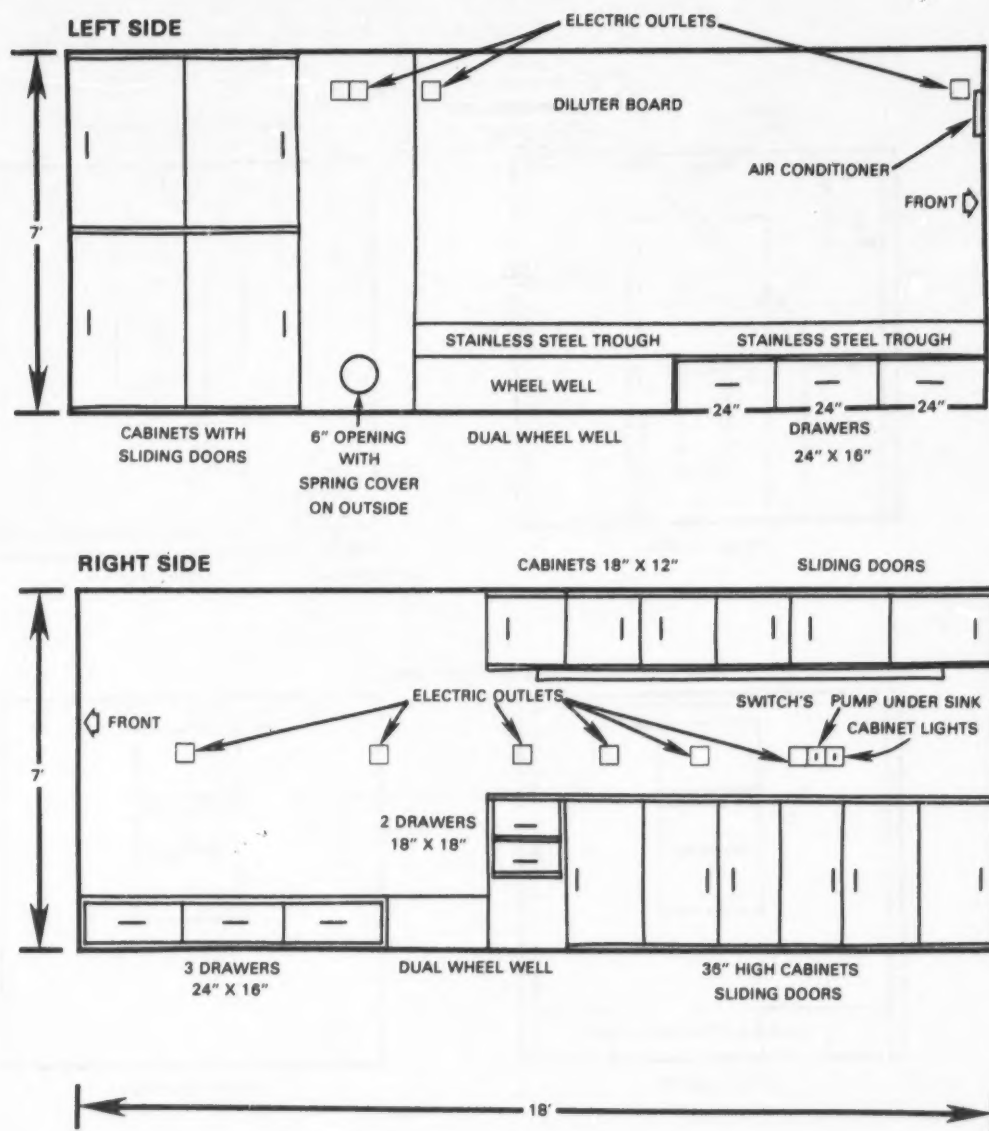


Figure 35. Mobile bioassay laboratory, tandem-axle trailer, internal views of side walls.

APPENDIX C

BIOASSAY MOBILE LABORATORY PLANS

2. FIFTH WHEEL TRAILER

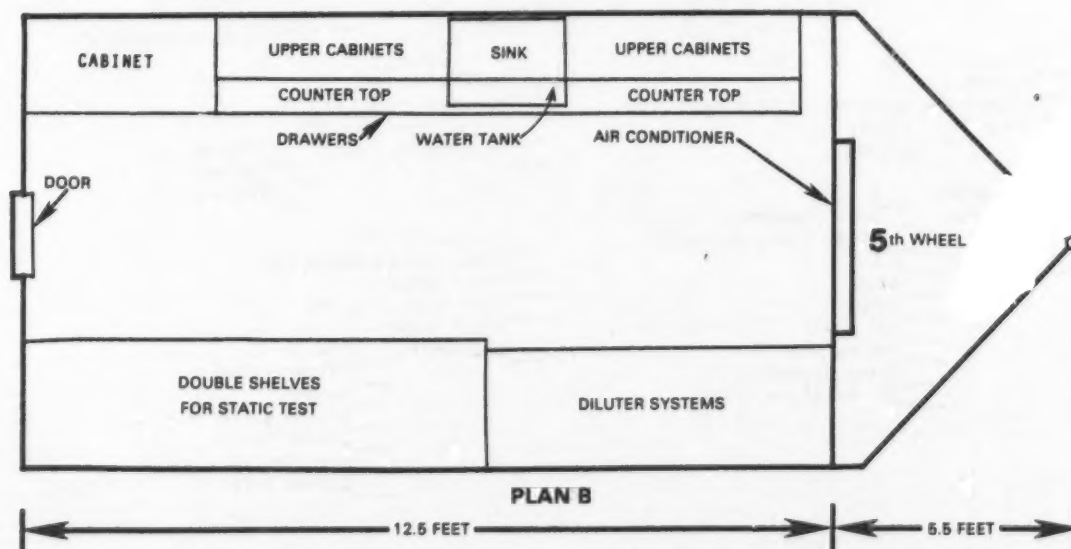
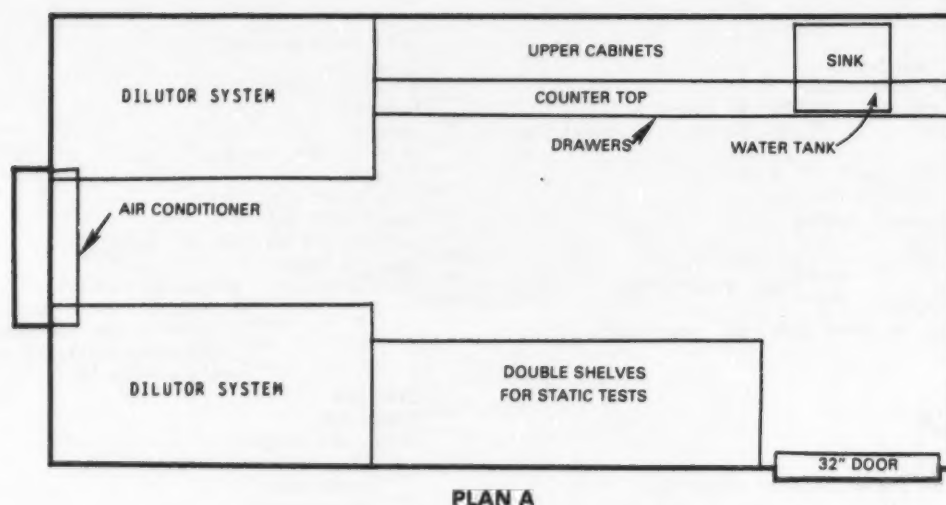


Figure 36. Mobile bioassay trailer, fifth-wheel trailer, internal view from above.

APPENDIX D
CHECK LISTS AND INFORMATION SHEETS¹

1. BIOASSAY FIELD EQUIPMENT LIST

Truck

☐ Boards
☐ Cinder blocks
☐ Drums: ☐ 500 gal nalgene
 ☐ 55 gal metal - diesel fuel
 ☐ 22 gal
☐ Gas can ☐ 5 gal
☐ Jacks
☐ Jumper cables
☐ Oil
☐ Pumps: ☐ (2) Homelite
 ☐ Hoses & couplings
☐ Shovels
☐ Spare tires (trailer, generator)

☐ Brine shrimp eggs
☐ Broom
☐ Brushes (wash)
☐ Buckets
☐ Camera
☐ Chlorine kit (w/chem)
☐ Cleanser
☐ Clip board (lg, sm)
☐ Cork borer set
☐ Culture dishes (200 mL, Daphnia)
☐ Daphnia food
☐ Data sheets: ☐ Bioassay (static)
 ☐ Bioassay (flow-thru)
 ☐ Diluter volume delivery
 ☐ Calibrator delivery sheet
 ☐ Daily events log

Trailer

☐ Acetone
☐ Aerators (battery operated)
☐ Air line: ☐ Clamps
 ☐ Aerators (battery operated)
 ☐ Air line: ☐ Clamps
 ☐ Stones
 ☐ Tubing
 ☐ Valves
☐ Alcohol
☐ Aluminum foil
☐ Alkalinity analysis (0.02 N H₂SO₄)
☐ Boots: ☐ safety
 ☐ wading
☐ Batteries ☐ D cell
☐ Beakers: ☐ 150 mL nalgene
 ☐ 200 mL glass (3 boxes)
☐ Bottles: ☐ D.O.
 ☐ Wash
 ☐ Sample
 ☐ VOA vials
 ☐ 500 mL plastic
 ☐ Glass organic
 ☐ Qt. w/teflon liner

☐ Dish pan
☐ Dish rack
☐ Dissolved oxygen:
 ☐ KCL membrane solution
 ☐ Membranes
 ☐ Meter (YSI)
 ☐ Probes
☐ Reagent: ☐ MnSO₄
 ☐ Alkaline azide
 ☐ H₂SO₄
 ☐ 0.0375 Na thiosulfate
☐ Starch
☐ Distilled H₂O
☐ Emergency road kit
☐ Enamel pans (lg, sm)
☐ Erlenmeyer flasks: ☐ 500 mL (2)
 ☐ 1000 mL
 ☐ 2000 mL
☐ Extension cords
☐ Fire extinguishers (2)
☐ First aid kit
☐ Fish nets, (lg, sm)
☐ Flash light
☐ Generator: ☐ Oil
 ☐ Filter - fuel
 ☐ Funnel
 ☐ Grease gun(wheels)
 ☐ Credit card
 ☐ Lock/key
 ☐ Siphon hose

¹Prepared by the staff of the Environmental Biology Section, Ecological Support Branch, Environmental Services Division, U.S. Environmental Protection Agency, Athens, Georgia 30613

BIOASSAY FIELD EQUIPMENT LIST (CONT.)

- ☐ Glass cutter
- ☐ Gloves (plastic)
- ☐ Graduated cylinders:
 - ☐ 25 mL, 50mL, 100mL
 - ☐ 250mL, 500mL, 1000mL, 2000mL
- ☐ Ground wire & rod
- ☐ Hand soap
- ☐ Hard hats
- ☐ Hardness analysis: ☐ Buffer
 - ☐ EDTA
 - ☐ indicator
- ☐ HCl (20%)
- ☐ Heaters: ☐ Aquarium
 - ☐ Space
- ☐ Hose: ☐ Clamps
 - ☐ Connectors
- ☐ Ice chests
- ☐ Jars: ☐ 750 mL (4 boxes)
 - ☐ 3 gal (glass) (1)
 - ☐ 5 gal (glass) (1)
 - ☐ Sample jugs (2)
- ☐ Kimwipes (1g,sm)
- ☐ Lab coats (2)
- ☐ Level
- ☐ Light 110 V
- ☐ Log book
- ☐ Magnetic stirrers: ☐ Lighted
 - ☐ Other
- ☐ Mop
- ☐ Paper towels
- ☐ Parachute cord
- ☐ Parafilm
- ☐ Pencils, pens
- ☐ pH: ☐ Meters, Orion
 - ☐ Meters, corning
 - ☐ Buffers, 4,7,10
 - ☐ Probes (extras)
- ☐ Pipets: ☐ Bulbs
 - ☐ Eyedroppers
 - ☐ Volumetric (1 mL, 5 mL, 10 mL)
- ☐ Plastic bags
- ☐ Quality assurance - SPCP
- ☐ Rain gear
- ☐ Reconstituted hard water
- ☐ Refractometer
- ☐ Respirators (cartridges)
- ☐ Rubber bands
- ☐ Ruler
- ☐ Safety glasses
- ☐ Safety manual
- ☐ Sample labels
- ☐ Scissors
- ☐ Screen bioassay cups
- ☐ Sea salts (Instant Ocean)
- ☐ Separatory funnels & racks
- ☐ Silent giants
- ☐ Silicon sealant
- ☐ Solenoids (spare)
- ☐ stainless steel tubing pieces
- ☐ Standard Methods Hand Book
- ☐ Stirring bars
- ☐ Stoppers (assorted)
- ☐ Submersible pumps: ☐ 1g, sm.
 - ☐ screens
- ☐ Super ice
- ☐ Tablets (paper)
- ☐ Tape: ☐ Cellophane
 - ☐ Color coded
 - ☐ Electrician
 - ☐ Masking
 - ☐ Nylon
- ☐ Thermometers: ☐ Dial
 - ☐ Glass
- ☐ Tools (lock/key)
- ☐ Tygon tubing, 1/8", 1/4", 3/8"
- ☐ Volumetric flasks (1000 mL, 2000 mL)
- ☐ WD40
- ☐ Weigh boats
- ☐ Wire tags

APPENDIX D

CHECK LISTS AND INFORMATION SHEETS

2. INFORMATION CHECK LIST FOR ON-SITE INDUSTRIAL
OR MUNICIPAL WASTE TOXICITY TEST.

1. PRE-TRIP INFORMATION

Facility Name: _____

Address: _____

Phone number: _____

Plant Representative(s): _____

Names, Titles, Addresses of Company Personnel:

A. To Receive Correspondence: _____

B. To Receive Carbons: _____

Date of Notification Letter: _____

State Making Notification and Arrangements: _____

Special Plant Safety/Security Requirements for EPA Personnel to Observe:

Local Accomodation Recommendations: _____

Directions to Plant: _____

Availability of Power Hookups (three 20-amp, 110-V Circuits): _____

Distance from Power Source to Trailer: _____ (Feet)

Trailer Location: _____

Possible Source of Dilution Water: _____

Major Products: _____

Raw Materials: _____

Name of Receiving Water: _____

Schedule of Plant Operation (continuous, weekdays only, etc.): _____

Treatment Steps: _____

Treatment Level (BPT, BAT, etc.): _____

Wastewater Retention Time by Lagoon or Treatment Step:

Lagoon Designation	Retention Time	
	Hours	Days
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Total Wastewater Retention Time: _____ Hours; _____ Days

Retention Time Determination: _____ Calculated; _____ Actual

Calculation method: _____

Description of Wastewater Tap Point: _____

Description of Outfall (surface, submerged diffuser, etc.): _____

Description of Other Waste Disposal Alternatives in Use (spray irrigation, deepwell, municipal discharge, etc.): _____

2. ON-SITE INFORMATION

Wastewater General Characteristics:

Color: _____

Odor: _____

Solids: _____

Other: _____

Serial Number(s) of Discharge(s) to be Tested: _____

Description of Receiving Water: _____ Uniflow; _____ Tidal;
_____ Approximate amplitude, feet

Color: _____

Odor: _____

Solids: _____

Salinity: High tide _____; Low tide _____

Other: _____

7Q10: _____; Ave. flow _____

Description of Receiving Water Zone of Dilution: _____

Location and Description of Water Sampling Point(s): _____

Fresh: _____

Salt: _____

Dilution Waste General Characteristics:

Color: _____

Odor: _____

Solids: _____

Other: _____

Description of Toxicity Test Anomalies (plant production changes, power failure, rain events, etc.):

Duration		Anomaly
Time & Date	Time & Date	
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Description of Plant maintenance: _____

DIAGRAM OF WASTEWATER TREATMENT FACILITIES:

3. FOLLOW-UP INFORMATION

Date of follow-up letter: _____

Wastewater Flow (data supplied by discharger):

<u>Week Prior to Testing</u>		<u>Week of Testing</u>	
<u>Date</u>	<u>Discharge (MGD)</u>	<u>Date</u>	<u>Discharge (MGD)</u>
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

Average Discharge (MGD): _____

Organisms Tested On-site or In-Lab:

<u>Species</u>	<u>Flow-thu</u>	<u>Static</u>	<u>Test</u>	<u>Dates</u>	<u>Results</u>
	<u>test</u>	<u>test</u>			
	<u>duration</u>	<u>duration</u>	<u>Location</u>		
	<u>(h)</u>	<u>(h)</u>			

Possible Recommended Action as a Result of These Findings:

APPENDIX D

CHECK LISTS AND INFORMATION SHEETS

3. DAILY EVENTS LOG

Date: _____

Page ____ of ____ Pages

Site: _____

Day #___ of Study

Initials: _____

Day #___ of Flow-through Test

Time:

Notes:

APPENDIX D
CHECK LISTS AND INFORMATION SHEETS
4. DILUTOR CALIBRATION FORM

Calibration Site: _____ Calibrator: _____
Diluter Number: _____ Date: _____

Effluent Concentration (%)	100.0	50.0	25.0	12.5	6.25	3.12	1.56
Dilution Water (mL)	0	500	750	875	938	969	984
Trial 1							
2							
3							
Average							
Effluent (mL)	1000	500	250	125	62	31	16
Trial 1							
2							
3							
Average							

Mixing Chamber (%): _____
Waste water (mL): _____
Dilution Water (mL): _____

	Dilution Water	Effluent
Vol (mL)		
Trial 1		
2		
3		
Average		

Remarks:

CHECK LISTS AND INFORMATION SHEETS

5. DAILY DILUTOR CALIBRATION CHECK

Industry: _____
Date: _____
Test Start Date: _____ Time: _____

USEPA
Athens, GA

Cycle Time (min)	Flow rate (ml/10 seconds) Dilution Water	Flow rate (ml/10 seconds) Wastewater	Turnover Time (min)
2.5	532	246	43
5.0	199	92	85
7.5	123	57	128
10.0	89	41	170
12.5	69	32	213
15.0 ^a	67	22	300

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APPENDIX E

COMPUTER PROGRAMS FOR CALCULATING THE LC50 AND 95% CONFIDENCE INTERVAL

(Prepared by James Dryer)

Five computer programs are described in this section. Four of the programs are provided for calculating the LC50 and 95% confidence intervals for acute toxicity test data.

- o Moving average-angle
- o Probit
- o Trimmed Spearman-Kärber
- o TOXDAT (Multi-method)

A fifth program (LC50) provides a statistical test to determine if LC50s from different samples are significantly different. All of the programs are written to run on a PDP 11/70 under IAS version 3.1. The program descriptions include information on the program language, examples of the use of the program with toxicity test data, and a complete program listing.

APPENDIX E

COMPUTER PROGRAMS FOR CALCULATING THE LC50 AND 95% CONFIDENCE INTERVAL

(Prepared by James Dryer)

1. MOVING AVERAGE-ANGLE PROGRAM

This program was written in BASIC PLUS 2, version 1.61, by James Dryer, EMSL-Cincinnati, and calculates the LC50 and 95% confidence interval for toxicity tests with fish and invertebrates. The program was based on a method published by Harris, "Confidence Limits for the LD50 using the Moving Average-Angle Method," Biometrics Vol. 3, Sept. 1959. The program is interactive, and prompts the user at each stage of input.

- NOTE: a. All concentrations are assumed to have the same number of fish at the start of the test.
b. For effluent toxicity tests, concentrations must be expressed as percent effluent.
c. Mortality is expressed as a percent, and not the actual number of animals that died.
d. Control information is not used in calculating the LC50.
e. Data must be entered from highest to lowest concentration
f. Use of a decimal is mandatory for concentration and mortality values, but is not used for the "number of concentrations."

EXAMPLE #1

A. INPUT

COMPUTER PROMPT:	Enter the number of fish at each concentration at the beginning of test
RESPONSE:	20
COMPUTER PROMPT:	Number of Concentrations (excluding the control)?
RESPONSE:	6
COMPUTER PROMPT:	Concentration Number 1?
RESPONSE:	100.0
COMPUTER PROMPT:	Mortality in number 1 concentration?
RESPONSE:	100.0
COMPUTER PROMPT:	Concentration Number 2?
RESPONSE:	50.0

COMPUTER PROMPT: Mortality in number 2 concentration?
RESPONSE: 80.0

The remaining prompts and responses are listed in abbreviated form below.

Concentration Number 3? = 25.0
Mortality in number 3 concentration? = 55.0

Concentration Number 4? = 12.5
Mortality in number 4 concentration? = 35.0

Concentration Number 5? = 6.25
Mortality in number 5 concentration? = 10.0

Concentration Number 6? = 3.12
Mortality in number 6 concentration? = 0.

B. OUTPUT

The printout for this set of data is as follows:

Confidence Limits (95%) for the LC50 using the Moving Average-Angle Method:

Effl. Conc. (%)	Log ₁₀ Conc.	Response (%)	Angle	Average Angle
100.	2.0000	100	83.5804	-
50.	1.69897	80	63.3525	64.9340
25.	1.39794	55	47.8692	49.1643
12.5	1.09691	35	36.2712	34.1918
6.25	.79588	10	18.4349	20.3753
3.12	.494155	0	6.41962	-

Lower Limit	15.08	1.1784
LC50	20.6165	1.31421
Upper Limit	29.8379	1.47477

G = 0.156^a

^aIf G is greater than 0.2, caution should be exercised in using the LC50 and confidence intervals. As G approaches 1.0, the LC50 and confidence limits become meaningless.)

EXAMPLE #2

A. INPUT:

The following concentrations and mortality data are input as described in Example #1, above.

Effluent Concentration	Mortality (%)
100.0	100.0
50.0	90.0
25.0	40.0
12.5	0.
6.25	0.
3.125	0.

B. OUTPUT:

The printout for this set of data is as follows:

Confidence Limits (95%) for the LC50 using the Moving Average-Angle Method

	Effl. Conc. (%)	Log ₁₀ Conc.	Response (%)	Angle	Average Angle
	100.	2.0000	100	83.5804	-
	50.	1.69897	90	71.0055	64.6058
	25.	1.39794	40	39.2315	38.8855
	12.5	1.09691	0	6.41962	17.3569
	6.25	.79588	0	6.41962	6.41962
	3.125	.49485	0	6.41962	-
Lower Limit	24.1671	1.38322			
LC50	29.4784	1.4965			
Upper Limit	35.2333	1.54695			

G = 0.053 (For the significance of "G," see p. 171)

COMPARISON OF LC50s FROM TWO TESTS

The moving average-angle program will also test for statistical significance between two LC50s, such as obtained from the data input in Examples #1 and #2, above. The comparison can be called up by inputting the value, "0.1," for "Number of Concentrations" when prompted by the program after completion of calculations for the second set of data.

A. INPUT

Enter the number of fish at each concentration at the beginning of test - 20

Number of Concentrations (excluding the control) - "0.1"

B. OUTPUT

The following output is produced by the program:

<u>Code #</u>	vs	<u>Code #</u>	<u>LC50(1)</u>	<u>LC50(2)</u>	<u>R_L</u>
1		2	29.4784	20.6165	-.228217

The two LC50s will be judged significantly different if:

$$(\text{Log}_{10} A - \text{Log}_{10} B) + R_L = 0$$

Where:

A = the concentration just below the LC50 value in one set of data (25% in Example #2), and

B = the like value for the other set (12.5% in Example #1).

In this case:

$$\begin{aligned} (\text{Log}_{10} A - \text{Log}_{10} B) + R_L &= (1.39794 - 1.09691) - 0.2282 \\ &= 0.07283 \end{aligned}$$

Since the sum of these values is greater than zero, the two LC50s are considered to be significantly different.

For a detailed explanation of the calculation of R_L , see Harris, (1959).

LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD

```

10 DIM D1(25),D2(25),R1(25),P1(25),A0(25),X(150),  &
X1(150),Y(150),Y1(150),K0(150),D7(150)
20 COM Z2,N,M,A,G,D4,Y2,D3,Y3,L4,L6
21 COM L5,D8,D9,R,R2,K,C1,M1,X0,Y4,D1,D2,R1,P1,A0,K5,X8
30 C4=.4342944819
40 C5=2.302585093
60 Z=1.96
61 INPUT" ENTER THE NUMBER OF FISH IN EACH TANK AT BEGINNING OF TEST",N
72 PRINT
80 M=0
90 Z2=Z**2
100 C1=6305.60224
110 FOR I = 1 TO 20
120 X(I)=0
130 A0(I)=0
140 NEXT I
150 E1=M
160 E=E1
170 A1=SQR(0.25/E1)
175 F=ATN(A1)/(SQR(1-A1**2))
189 P1(1)=F
190 P0=P1(1)*180/3.14159265
200 P1(1)=0
210 P9=90-P0
220 K9=1
230 N=M+1
240 K=3
244 PRINT
245 PRINT " N O T E . . . 'TO END' , TYPE IN 999 FOR CODE"
246 PRINT
250 INPUT "CODE - (UP TO 4 DIGITS)",K0(M)
251 IF K0(M)=0 THEN 250
252 IF K0(M)=999 THEN 3440
255 PRINT
258 PRINT "IF ALL DATA HAS BEEN ENTERED , TYPE IN"
259 PRINT "0.1 FOR 'NUMBER OF CONCENTRATIONS'."
260 INPUT "NUMBER OF CONCENTRATIONS",M1
261 IF M1=0 THEN 260
262 IF M1=0.1 THEN 1261
265 PRINT
280 IF K9=1 THEN 283
283 PRINT " PLEASE USE A DECIMAL POINT IN CONCENTRATION"
284 PRINT " AND PERCENT MORTALITY"
285 PRINT
290 FOR I=1 TO M1
295 PRINT "CONCENTRATION NUMBER ";I;" = "
300 INPUT D1(I)
301 IF D1(I)=0 THEN 300

```


LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD (CONT.)

```

308 PRINT "MORTALITY (IN PERCENT) IN NUMBER ";I;"CONCENTRATION = "
310 INPUT R1(I)
311 R1(I)=R1(I)/100
330 NEXT I
340 K9=2
350 GO TO 480
360 FOR I = 1 TO M1
370 PRINT "CONCENTRATION NUMBER ";I;" = "
371 INPUT D1(I)
372 IF D1(I)=0 THEN 370
380 PRINT "MORTALITY (IN PERCENT) IN NUMBER ";I;"CONCENTRATION = "
381 INPUT R1(I)
382 R1(I)=R1(I)/100
383 IF R1(I)=0 THEN 380
385 IF R1(I)=.001 THEN 387
386 GO TO 390
387 R1(I)=0
390 NEXT I
410 K9=3
420 GO TO 480
430 FOR I = 1 TO M1
440 PRINT "CONCENTRATION NUMBER ";I;" = "
441 INPUT D1(I)
442 IF D1(I)=0 THEN 440
450 PRINT "MORTALITY (IN PERCENT) IN NUMBER ";I;"CONCENTRATION = "
451 INPUT R1(I)
452 R1(I)=R1(I)/100
453 IF R1(I)=0 THEN 450
454 IF R1(I)=.001 THEN 456
455 GO TO 460
456 R1(I)=0
460 NEXT I
470 K9=1
480 FOR I = 1 TO M1
490 IF (R1(I))<>0 THEN 520
500 P1(I)=P0
510 GO TO 580
520 IF (R1(I)-1)<0 THEN 560
530 IF (R1(I)-1)>0 THEN 580
540 P1(I)=P9
550 GO TO 580
560 A1=SQR(R1(I))
570 F31=A1+.1666666667*A1**3+.075*A1**5+.0446428571*A1**7
571 F32=.0303819444*A1**9+.022372159*A1**11+.0173527644*A1**13
572 F34=.00976160952*A1**19+.0083903358*A1**21+.00731252587*A1**23
573 F33=.0139648437*A1**15+.0115518008*A1**17
576 F3=F31+F32+F33+F34
577 F4=F3*180/PI

```

LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD (CONT.)

```

579 P1(I)=F4
580 D2(I)=LOG(D1(I))*C4
590 R1(I)=100*R1(I)
600 NEXT I
610 PRINT "CONFIDENCE LIMITS FOR THE LC(50) USING THE"
611 PRINT "      MOVING AVERAGE - ANGLE METHOD"
621 PRINT "CONC      LOG CONC      MORTALITY      ANGLE      AVERAGE"
639 M2=M1-1
640 C=3
650 K6=2
660 FOR I = K6 TO M2
670 A0(I)=(P1(I-1)+P1(I)+P1(I+1))/3
680 NEXT I
690 IF (A0(M2)-45)<0 THEN 850
700 IF (P1(M1)-45)>0 THEN 2560
710 C8=2
720 Y1(M)=A0(M2)
730 X1(M)=D2(M2)
740 J=M1
750 FOR I = 1 TO M1
760 IF (P1(J)-45)>=0 THEN 820
770 J=J-1
780 NEXT I
790 X(M)=D2(1)
800 Y(M)=P1(1)
810 GO TO 1090
820 X(M)=D2(J+1)
830 Y(M)=P1(J+1)
840 GO TO 1090
850 IF (A0(K6)-45) > 0 THEN 990
860 IF (P1(1)-45)<0 THEN 2560
870 C8=1
880 Y(M)=A0(K6)
890 X(M)=D2(K6)
900 J=M1
910 FOR I = 1 TO M1
920 IF (P1(J)-45)>=0 THEN 960
930 J=J-1
940 NEXT I
950 GO TO 2560
960 X1(M)=D2(J)
970 Y1(M)=P1(J)
980 GO TO 1090
990 J=M2
1000 FOR I = K6 TO M2
1010 IF (A0(J)-45)>0 THEN 1040
1020 J=J-1
1030 NEXT I

```

LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD (CONT.)

```

1040 C8=3
1050 X1(M)=D2(J)
1060 X(M)=D2(J+1)
1070 Y1(M)=A0(J)
1080 Y(M)=A0(J+1)
1090 D4=X1(M)-X(M)
1100 Y2=Y1(M)-Y(M)
1110 A=(45-Y(M))/Y2
1120 D7(M)=X(M)+A*D4
1130 A3=D7(M)*C5
1140 L5=EXP(A3)
1150 X8=X(M)
1170 GOSUB 2740
1180 IF C8=3 THEN 1240
1190 IF C8=2 THEN 1220
1200 GO SUB 2870
1210 GO TO 1242
1220 GOSUB 3030
1230 GO TO 1242
1240 GOSUB 3190
1242 PRINT
1250 PRINT L4,"      ",D8,"      LOWER LIMIT"
1251 PRINT L5,"      ",D7(M),"      LC(50)"
1252 PRINT L6,"      ",D9,"      UPPER LIMIT (95%)"
1253 PRINT "G = ",G
1259 PRINT
1260 GO TO 230
1261 PRINT\PRINT
1271 PRINT "CODE#      VS      CODE#      LC50(1)      LC50(2)      R"
1280 N,L=M-1
1300 FOR I = 1 TO M STEP 3
1310 IF (D7(I)-D7(I+1))>=0 THEN 1430
1320 D4=X1(I)-X(I)
1330 D3=X1(I+1)-X(I+1)
1340 Y2=Y1(I)-Y(I)
1350 Y3=Y1(I+1)-Y(I+1)
1360 X0=X(I+1)-X(I)
1370 Y4=Y(I)-Y(I+1)
1380 X7=D7(I+1)
1390 X2=D7(I)
1420 GO TO 1530
1430 D4=X1(I+1)-X(I+1)
1440 D3=X1(I)-X(I)
1450 Y2=Y1(I+1)-Y(I+1)
1460 Y3=Y1(I)-Y(I)
1470 X0=X(I)-X(I+1)
1480 Y4=Y(I+1)-Y(I)
1490 X7=D7(I)

```

LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD (CONT.)

```

1500 X2=D7(I+1)
1530 GOSUB 3350
1540 X3=X7*C5
1550 X4=X2*C5
1560 X5=EXP(X3)
1570 X6=EXP(X4)
1580 PRINT K0(I),K0(I+1),X5,X6,R2
1590 IF (D7(I)-D7(I+2))<0 THEN 1600
1595 GO TO 1710
1600 D4=X1(I)-X(I)
1610 D3=X1(I+2)-X(I+2)
1620 Y2=Y1(I)-Y(I)
1630 Y3=Y1(I+2)-Y(I+2)
1640 X0=X(I+2)-X(I)
1650 Y4=Y(I)-Y(I+2)
1660 X7=D7(I+2)
1670 X2=D7(I)
1700 GO TO 1810
1710 D4=X1(I+2)-X(I+2)
1720 D3=X1(I)-X(I)
1730 Y2=Y1(I+2)-Y(I+2)
1740 Y3=Y1(I)-Y(I)
1750 X0=X(I)-X(I+2)
1760 Y4=Y(I+2)-Y(I)
1770 X7=D7(I)
1780 X2=D7(I+2)
1810 GOSUB 3350
1820 X3=X7*C5
1830 X4=X2*C5
1840 X5=EXP(X3)
1850 X6=EXP(X4)
1860 PRINT K0(I),K0(I+2),X5,X6,R2
1870 IF (D7(I+1)-D7(I+2))<0 THEN 1880
1875 GO TO 1990
1880 D4=X1(I+1)-X(I+1)
1890 D3=X1(I+2)-X(I+2)
1900 Y2=Y1(I+1)-Y(I+1)
1910 Y3=Y1(I+2)-Y(I+2)
1920 X0=X(I+2)-X(I+1)
1930 Y4=Y(I+1)-Y(I+2)
1940 X7=D7(I+2)
1950 X2=D7(I+1)
1980 GO TO 2090
1990 D4=X1(I+2)-X(I+2)
2000 D3=X1(I+1)-X(I+1)
2010 Y2=Y1(I+2)-Y(I+2)
2020 Y3=Y1(I+1)-Y(I+1)
2030 X0=X(I+1)-X(I+2)

```

LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD (CONT.)

```

2040 Y4=Y(I+2)-Y(I+1)
2050 X7=D7(I+1)
2060 X2=D7(I+2)
2090 GOSUB 3350
2100 X3=X7*C5
2110 X4=X2*C5
2120 X5=EXP(X3)
2130 X6=EXP(X4)
2140 PRINT K0(I+1),K0(I+2),X5,X6,R2
2150 NEXT I
2160 M3=M-6
2170 L1=1
2180 L2=3
2190 FOR J= L1 TO L2
2200 J1=J+3
2210 FOR I = J1 TO M STEP 3
2220 IF (D7(J)-D7(I))<0 THEN 2230
2225 GO TO 2340
2230 D4=X1(J)-X(J)
2240 D3=X1(I)-X(I)
2250 Y2=Y1(J)-Y(J)
2260 Y3=Y1(I)-Y(I)
2270 X0=X(I)-X(J)
2280 Y4=Y(J)-Y(I)
2290 X7=D7(I)
2300 X2=D7(J)
2330 GO TO 2440
2340 D4=X1(I)-X(I)
2350 D3=X1(J)-X(J)
2360 Y2=Y1(I)-Y(I)
2370 Y3=Y1(J)-Y(J)
2380 X0=X(J)-X(I)
2390 Y4=Y(I)-Y(J)
2400 X7=D7(J)
2410 X2=D7(I)
2440 GOSUB 3350
2450 X3=X7*C5
2460 X4=X2*C5
2470 X5=EXP(X3)
2480 X6=EXP(X4)
2490 PRINT K0(J),K0(I),X5,X6,R2
2500 NEXT I
2510 NEXT J
2520 IF (L1-M3)<0 THEN 2530
2525 GO TO 2740
2530 L1=L1+3
2540 L2=L2+3
2550 GO TO 2190

```


LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD (CONT.)

```

2560 PRINT "AVERAGE AND TRANSFORMED ANGLES DO NOT BRACKET 45 DEGREES"
2570 GO TO 230
2580 PRINT R1(I),"HAS EXCEEDED 100 PERCENT"
2590 GO TO 230
2740 PRINT D1(1),D2(1),R1(1),P1(1)
2750 J=M1-1
2760 FOR I = 2 TO J
2770 PRINT D1(I),D2(I),R1(I),P1(I),A0(I)
2780 NEXT I
2790 PRINT D1(M1),D2(M1),R1(M1),P1(M1)
2800 RETURN
2870 C5=2.302585093
2880 G=C1/(E*C*Y2**2)
2890 G1=1-G
2900 W=(SQR(G)/G1)*(SQR(A**2+G1/2))
2910 A5=A/G1
2920 D8=X8+D4*(A5-W)
2930 D9=X8+D4*(A5+W)
2940 X9=D8*C5
2950 L4=EXP(X9)
2960 X9=D9*C5
2970 L6=EXP(X9)
2980 RETURN
3030 C5=2.302585093
3040 G=C1/(E*C*Y2**2)
3050 G1=1-G
3060 A5=(A-G)/G1
3070 W=(SQR(G)/G1)*SQR((A-1)**2+G1/2)
3080 D8=(A5-W)*D4+X8
3090 D9=(A5+W)*D4+X8
3100 X9=D8*C5
3110 L4=EXP(X9)
3120 X9=D9*C5
3130 L6=EXP(X9)
3140 RETURN
3190 C5=2.302585093
3200 G=C1/(E*C**2*Y2**2)
3210 G1=1-G
3220 A5=(A-G/2)/G1
3230 W=(SQR(G)/G1)*SQR((A-.5)**2+G1*((2*C-1)/4))
3240 D8=X8+D4*(A5-W)
3250 D9=X8+D4*(A5+W)
3260 X9=D8*C5
3270 L4=EXP(X9)
3280 X9=D9*C5
3290 L6=EXP(X9)
3300 RETURN
3350 D=D3**2+D4**2

```

LISTING OF COMPUTER PROGRAM FOR MOVING AVERAGE-ANGLE METHOD (CONT.)

```
3360 V2=1641.4/(D*E*C**2)
3370 B1=(D3*Y3+D4*Y2)/D
3380 H=Z2*V2/B1**2
3390 H1=1-H
3400 V1=1641.4/(E*C)
3410 R2=(Y4-SQR(Z2*H1*V1+H*Y4**2))/(H1*B1)
3420 R=X0+R2
3430 RETURN
3440 END
```

APPENDIX E

COMPUTER PROGRAMS FOR CALCULATING THE LC50 AND 95% CONFIDENCE INTERVAL

2. PROBIT PROGRAM

This program is written in FORTRAN PLUS IV v.3, and calculates the LC50 and 95% confidence limits using the Probit transformation. It will also conduct a test to determine if two sets of toxicity test data are significantly different.

The program was written by You Yen Yang, a statistician formerly with the USEPA at the Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio, and was modified by James Dryer.

- NOTE:
- a. Control information is not used in calculating the LC50.
 - b. Data must be input from the HIGHEST to the LOWEST concentration.
 - c. Input the NUMBER of animals that died, NOT THE PERCENT.
 - d. Decimals must be used for data on the concentration and mortality, but not for the "Number of Concentrations."

EXAMPLE #1

A. INPUT

Number of Concentrations (excluding the control) = 6

Concentration = 100. (%)

No. of animals in this concentration at the start of the test = 20.

No. of animals that died at this concentration = 20.

Concentration = 50.

No. of Animals in this concentration at the start of the test = 20.

No. of Animals that died at this concentration = 16.

Concentration = 25.

No. of animals in this concentration at the start of the test = 20.

No. of Animals that died at this concentration = 11.

Concentration = 12.5

No. of Animals in this concentration at the start of the test = 20.

No. of Animals that died at this concentration = 7.

Concentration = 6.25

No. of Animals in this concentration at the start of the test = 20.

No. of Animals that died at this concentration = 2.

Concentration = 3.125

No. of Animals in this concentration at the start of the test = 20.

No. of Animals that died at this concentration = 0.

B. OUTPUT:

The printout for this set of data is as follows:

PROBIT ANALYSIS

	CONC	LOG(10) CONC	NUMBER OF OBSERVATIONS	NUMBER OF RESPONSES	RATIO
1	100.00	2.00000	20	20	1.0000
2	50.00	1.69897	20	16	0.8000
3	25.00	1.39794	20	11	0.5500
4	12.50	1.09691	20	7	0.3500
5	6.25	0.79588	20	2	0.1000
6	3.12	0.49485	20	0	0.0000

INTERCEPT	=	1.49849
SLOPE	=	2.67833
T-VALUE FOR B	=	6.76726 WITH 4 DF
WEIGHTED MEAN LOG(10) CONC	=	1.29800
WEIGHTED MEAN PROBIT Y	=	4.97497
CHI SQUARE FOR LINEARITY	=	2.02629 WITH 4 DF
P = 0.731		

LC50	=	20.29309
UPPER LIMIT OF LC50	=	26.53896 (AT .95)
LOWER LIMIT OF LC50	=	15.57846

WEIGHTED SUM OF SD OF X	=	6.38408
WEIGHTED N	=	43.71228
G	=	0.08389

For information on the significance of "G" see comments p. 171.

EXAMPLE #2

A. INPUT:

The second set of test data listed below, is input as described for the previous example.

Effluent Concentration (%)	Number Responding
100.0	20
50.0	18
25.0	8
12.5	0
6.25	0
3.125	0

B. OUTPUT:

The printout for this set of data is listed below:

PROBIT ANALYSIS					
	LOG(10) CONC	NUMBER OF OBSERVATIONS	NUMBER OF RESPONSES	RATIO	
1	100.00	20	20	1.0000	
2	50.00	20	18	0.9000	
3	25.00	20	8	0.4000	
4	12.50	20	0	0.0000	
5	6.25	20	0	0.0000	
6	3.12	20	0	0.0000	
INTERCEPT = -3.71367					
SLOPE = 5.96065					
T-VALUE FOR B = 4.96081 WITH 4 DF					
WEIGHTED MEAN LOG(10) CONC = 1.46267					
WEIGHTED MEAN PROBIT Y = 5.00478					
CHI SQUARE FOR LINEARITY = 0.64423 WITH 4 DF					
P = 0.958					
LC50 = 28.96448					
UPPER LIMIT OF LC50 = 34.79466 (AT .95)					
LOWER LIMIT OF LC50 = 24.09461					
WEIGHTED SUM OF SQ OF X = 0.69243					
WEIGHTED N = 20.12352					
S = 0.15615					

For information on the significance of "G" see comments p. 171.

LISTING OF COMPUTER PROGRAM FOR PROBIT METHOD

```

      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION X(25),RN(25),WPRO(25),Y(25),SXX(2),SXY(2),A(3),B(3),
      1SY(2),SX(2),SY(2),SWN(2),XBAR(2),YBAR(2),EP(25),WN(25),
      2RP(25),U(25),R(25),P(25),SN(2),TT(30),TITLE(20),PR(10),KJ(10)
      INTEGER ANSWER,COMP
      DATA TT/12.706,4.303,3.182,2.776,2.571,2.444,2.365,2.306,2.262,2.2
      128,2.201,2.179,2.16,2.145,2.131,2.12,2.11,2.101,2.093,2.086,2.08,
      22.074,2.069,2.064,2.06,2.056,2.052,2.048,2.045,2.042/
      DATA PR/3.36,3.72,3.96,4.16,4.33,5.67,5.84,6.04,6.28,6.64/
      DATA KJ/5,10,15,20,25,75,80,85,90,95/

C
C
C  PROBIT ANALYSIS
C
      TYPE*, '          PROBIT - ANALYSIS'
      TYPE*, ' '
      1 VARYS= 0.
      STXX = 0.
      STXY = 0.
      STYY = 0.
      SUMX = 0.
      SUMY = 0.
      STWN = 0.
      SUMN = 0.
      CHI = 0.
      ZZZZ = 0.
      I=0
      30 I=I+1
      IF(I.EQ.3) GO TO 13
      TYPE 45
      45 FORMAT(' NUMBER OF CONCENTRATIONS = ',%)
      ACCEPT 44,M
      44 FORMAT(I2)
      DO 5 J=1,M
      TYPE 880
      880 FORMAT(' CONCENTRATION = ',%)
      ACCEPT 881,U(J)
      881 FORMAT(F5.2)
      TYPE 882
      882 FORMAT(' NO. OF ANIMALS AT START OF TEST IN THIS CONCEN. = ',%)
      ACCEPT 883,RN(J)
      883 FORMAT(F2.0)
      TYPE 884
      884 FORMAT(' NO. OF ANIMALS RESPONDING IN THIS CONCENTRATION = ',%)
      ACCEPT 885,R(J)
      885 FORMAT(F2.0)
      TYPE*, ' '

```

LISTING OF COMPUTER PROGRAM FOR PROBIT METHOD (CONT.)

```

TYPE*, '
X(J)=DLOG10(U(J))
RP(J)=R(J)/RN(J)
IF(RP(J))3,3,4
3 EP(J)=0.
  GO TO 5
4 T=DSQRT(DLOG(1./RP(J)**2))
  EP(J)=T-(2.515517+.802853*T+.010328*T*T)/(1.+1.432788*T+.189269*T*
  1T+.001308*T**3)
  EP(J)=5.-EP(J)
5 CONTINUE
  SSX = 0.
  SSY = 0.
  XY = 0.
  SMX = 0.
  SHYX = 0.
  SHY = 0.
  DO 6 J=1,M
    SSX=SSX+X(J)**2
    XY=XY+X(J)*EP(J)
    SMX=SMX+X(J)
6 SHY=SHY+EP(J)
  FM=M
  B(I)=(XY-SMX*SHY/FM)/(SSX-SMX**2/FM)
  A(I)=(SHY-B(I)*SMX)/FM
7 SXX(I) = 0.
  SYY(I) = 0.
  SXY(I) = 0.
  SX(I) = 0.
  SY(I) = 0.
  SN(I) = 0.
  SWN(I) = 0.
  DO 10 J=1,M
    Y(J)=A(I)+B(I)*X(J)
    YY=Y(J)
    CALL NID(YY,Z,PP)
    P(J)=PP
    IF(Y(J)-5.)9,8,8
8 P(J)=1.-P(J)
9 Q=1.-P(J)
  U=Z*Z/(P(J)*Q)
  UN(J)=RN(J)*U
  WPRO(J)=Y(J)-(P(J)-RP(J))/Z
  SXX(I)=SXX(I)+X(J)**2*UN(J)
  SYY(I)=SYY(I)+WPRO(J)**2*UN(J)
  SXY(I)=SXY(I)+X(J)*WPRO(J)*UN(J)
  SX(I)=SX(I)+X(J)*UN(J)
  SY(I)=SY(I)+WPRO(J)*UN(J)

```

LISTING OF COMPUTER PROGRAM FOR PROBIT METHOD (CONT.)

```

      SWN(I)=SWN(I)+WN(J)
10  CONTINUE
      B(I)=(SXY(I)-SX(I)*SY(I)/SWN(I))/(SXX(I)-SX(I)**2/SWN(I))
      A(I)=(SY(I)-B(I)*SX(I))/SWN(I)
      DO 11 J=1,M
      D=DABS(A(I)+B(I)*X(J)-Y(J))
      IF((D-.0000001).GT.0) GO TO 7
11  CONTINUE
      WRITE(5,500)
500  FORMAT('                                PROBIT ANALYSIS ')
      WRITE(5,501)
501  FORMAT('                                ')
      WRITE(5,504)
      WRITE(5,502)
502  FORMAT('                                LOG(10)  NUMBER OF  NUMBER OF  RATIO')
      WRITE(5,503)
503  FORMAT('          CONC      CONC  OBSERVATIONS  RESPONSES')
      WRITE(5,504)
504  FORMAT(' ')
      TYPE*, ' '
      XBAR(I)=SX(I)/SWN(I)
      YBAR(I)=SY(I)/SWN(I)
      WWWW=SXY(I)-SX(I)*SY(I)/SWN(I)
      XXXX=SXX(I)-SX(I)**2/SWN(I)
      YYYY=SY(I)-SY(I)**2/SWN(I)
      ZZZZ=ZZZZ+WWWW**2/XXXX
      STYY=STYY+YYYY
      STXX=STXX+XXXX
      STXY=STXY+WWWW
      STUN=STUN+SWN(I)
      YYYY=YYYY-WWWW**2/XXXX
      CHI=CHI+YYYY
      VARYS=VARYS+1./SWN(I)
      SUNX=SUNX+SX(I)
      SUMY=SUMY+SY(I)
      SUMN=SUMN+FM
      MMMM=M-2
      AM=(5.-A(I))/B(I)
      ED=10.**AM
      DF=MMMM
      CHIP=CHIPR(DF,YYYY)
      IF(CHIP-.05)16,16,15
15  T=1.96
      S=1.
      GO TO 20
16  IF(DF-30.)17,17,18
17  T=TT(MMMM)
      GO TO 19

```

LISTING OF COMPUTER PROGRAM FOR PROBIT METHOD (CONT.)

```

18 T=2.0
19 S=YYYY/DF
20 G=S*T/(B(I)**2*XXX)
   ST=B(I)*DSQRT(XXX)/S
   IF(G-1.)22,21,22
21 G=.99999
22 CON=(1.-G)/SUM(I)+(AM-XBAR(I))**2/XXX
   CON=CON*S
   IF(CON)24,23,23
23 CON=DSQRT(CON)
   FED=AM+G*(AM-XBAR(I))/(1.-G)-CON*T/(B(I)*(1.-G))
   UED=AM+G*(AM-XBAR(I))/(1.-G)+CON*T/(B(I)*(1.-G))
   UED=10.**UED
   FED=10.**FED
   GO TO 25
24 UED=0.
   FED=0.
25 DO 28 J=1,M
   N=RN(J)
   K=R(J)
   CON=(1.-G)/SUM(I)+(X(J)-XBAR(I))**2/XXX
   IF(CON)27,26,26
26 CON=DSQRT(CON)
   PU=X(J)+G*(X(J)-XBAR(I))/(1.-G)+CON*T/(B(I)*(1.-G))
   PL=X(J)+G*(X(J)-XBAR(I))/(1.-G)-CON*T/(B(I)*(1.-G))
   PU=A(I)+PU*B(I)
   PL=A(I)+PL*B(I)
   CALL NID(PU,Z,Q)
   IF(PU.GT.5.)Q=1-Q
   CALL NID(PL,Z,QQ)
   IF(PL.GT.5.)QQ=1.-QQ
   GO TO 28
27 Q=0.
   QQ=0.
28 WRITE(5,29)J,U(J),X(J),N,K,RP(J)
29 FORMAT(I3,F9.2,F9.5,5X,I4,9X,I4,F10.4)
   WRITE(5,47)A(I)
   WRITE(5,210)B(I)
   WRITE(5,211)ST,MMM
   WRITE(5,212)XBAR(I)
   WRITE(5,213)YBAR(I)
   WRITE(5,214)YYYY,MMM,CHIP
   WRITE(5,215)ED
   WRITE(5,216)UED
   WRITE(5,217)FED
   WRITE(5,200)XXX
   WRITE(5,218)SUM(I)
   WRITE(5,219)G

```

LISTING OF COMPUTER PROGRAM FOR PROBIT METHOD (CONT.)

```

      IF (COMP.EQ.'YES') GO TO 13
      TYPE*, ' IF YOU WOULD LIKE TO COMPARE ANOTHER SET OF DATA TO THIS'
      TYPE*, ' SET, TYPE IN "YES" FOR "COMPARE?". OTHERWISE, TYPE "NO" '
      TYPE 651
651  FORMAT(' COMPARE ?',%)
      ACCEPT 652,COMP
652  FORMAT(20A2)
      IF (COMP.EQ.'NO') GO TO 556
      GO TO 30
13  TYPE*, '***WHEN THE CONFIDENCE LIMITS TO P AND LC50 ARE 0,'
      TYPE*, ' THEY ARE NOT REALLY 0, BUT THEY ARE NOT ESTIMATABLE.'
      TYPE*, 'G IS (BY FINNEY S NOTATION) TOO BIG.***'
      DF=SUMN-4.
      ND=SUMN-4.
      CHIP=CHIPR(DF,CHI)
      IF (CHIP-.05) 32,32,35
32  S=CHI/DF
      IF (DF-30.) 33,33,34
33  IDF=DF
      T=TT(IDF)
      GO TO 36
34  T=2.
      GO TO 36
35  S=1.
      T=1.96
36  CHI1=ZZZZ-STXY**2/STXX
      PP=CHIPR(1.,CHI1)
37  B(3)=STXY/STXX
      VARB=S/STXX
      VARYS=VARYS*S
      POTE=XBAR(1)-XBAR(2)-(YBAR(1)-YBAR(2))/B(3)
      POTEN=10.**POTE
      G=S*T/(B(3)**2*STXX)
      D=POTE-XBAR(1)+XBAR(2)
      IF (G-1.) 39,38,39
38  G=.999999
39  E=(1.-G)*VARYS+D*D*STXX
      IF (E) 41,40,40
40  E=T*DSQRT(E)/B(3)
      FLOW=XBAR(1)-XBAR(2)+(D-E)/(1.-G)
      UPPER=XBAR(1)-XBAR(2)+(D+E)/(1.-G)
      UPPER=10.**UPPER
      FLOW=10.**FLOW
      GO TO 42
41  UPPER=0.
      FLOW=0.
42  WRITE(5,43)CHI,ND,CHIP
43  FORMAT(10X,'CHI SQ. FOR LINEARITY',F12.4,' WITH ',I4,' DF,P=',F8.4)
      WRITE(5,201)CHI1,PP
201  FORMAT(10X,'CHI SQ FOR PARALLELISM',F12.4,' WITH 1 DF,P =',F8.4)

```


LISTING OF COMPUTER PROGRAM FOR PROBIT METHOD (CONT.)

```

WRITE(5,202)B(3)
202 FORMAT(10X,'POOLED SLOPE',12X,F12.4)
WRITE(5,203)VARB
203 FORMAT(10X,'VARIANCE FOR B',10X,F12.4)
WRITE(5,204)VARYS
204 FORMAT(10X,'VAR(YBAR(S)-YBAR(T))',4X,F12.4)
WRITE(5,205)POTEN
205 FORMAT(10X,'RELATIVE POTENCY R',6X,F12.4)
WRITE(5,206)UPPER
206 FORMAT(10X,'UPPER LIMIT OF R (AT .95)',F11.4)
WRITE(5,207)FLOW
207 FORMAT(10X,'LOWER LIMIT OF R (AT .95)',F11.4)
47 FORMAT(/,14X,'INTERCEPT',18X,'=',F14.5)
210 FORMAT(14X,'SLOPE',22X,'=',F14.5)
211 FORMAT(14X,'T-VALUE FOR B',14X,'=',F14.5,' WITH',I2,' DF')
212 FORMAT(14X,'WEIGHTED MEAN LOG(10) CONC =',F14.5)
213 FORMAT(14X,'WEIGHTED MEAN PROBIT Y',5X,'=',F14.5)
214 FORMAT(14X,'CHI SQUARE FOR LINEARITY =',F14.5,' WITH',I2,' DF'
1/,14X,' P = ',F5.3)
215 FORMAT(/,14X,'LC50',23X,'=',F14.5)
216 FORMAT(14X,'UPPER LIMIT OF LC50',8X,'=',F14.5,' (AT .95)')
217 FORMAT(14X,'LOWER LIMIT OF LC50',8X,'=',F14.5)
200 FORMAT(/,14X,'WEIGHTED SUM OF SQ OF X',4X,'=',F14.5)
218 FORMAT(14X,'WEIGHTED N',17X,'=',F14.5)
219 FORMAT(14X,'G',26X,'=',F14.5,//////)
IF(PF.GT..05)GO TO 1
DO 51 J=1,10
DO 48 I=1,2
48 EP(I)=XBAR(I)+(PR(J)-YBAR(I))/B(I)
POTE=EP(1)-EP(2)
P(J)=10.**POTE
D=POTE-XBAR(1)+XBAR(2)
E=(1.-G)*VARYS+D*D/STXX
IF(E)49,50,50
49 X(J)=0.
Y(J)=0.
GO TO 51
50 E=T*DSQRT(E)/B(3)
X(J)=XBAR(1)-XBAR(2)+(D-E)/(1.-G)
Y(J)=XBAR(1)-XBAR(2)+(D+E)/(1.-G)
X(J)=10.**X(J)
Y(J)=10.**Y(J)
51 CONTINUE
WRITE(5,52)
52 FORMAT(1H1 ,14X,'THE RELATIVE POTENCY AND ITS .95 LIMITS AT'
1/1H ,14X,'DIFFERENT PERCENTILES WHEN PARALLELISM WAS REJECTED',//
21H ,31X,'R(L)',7X,'R',6X,'R(U)',/)
DO 53 J=1,10
IF (J.EQ.6)WRITE(5,54)
53 WRITE(5,55)XJ(J),X(J),P(J),Y(J)

```

LISTING OF COMPUTER PROGRAM FOR PROBIT METHOD (CONT.)

```

54 FORMAT(/1H )
55 FORMAT(20X,I6,'Z',3F9.4)
   IF(ANSWER.EQ.'YES')GO TO 56
556 TYPE*, ' DO YOU HAVE MORE DATA TO ANALYZE ?'
   TYPE 653
653 FORMAT(' TYPE IN "YES" OR "NO" - ',8)
   ACCEPT 654,ANSWER
654 FORMAT(20A2)
   IF (ANSWER.EQ.'YES') GO TO 1
56 STOP
   END
   SUBROUTINE MID(Y,Z,P)
   IMPLICIT REAL*8(A-H,O-Z)
   E=DABS (Y-5.)
   T=1./(1+.2316419*E)
   Z=0.3989422804*DEXP(-0.5*E*E)
   P=Z*(.31938153*T-.356563782*T+1.781477937*T**3-1.821255978*T*
1*4+1.330274429*T**5)
   RETURN
   END
   FUNCTION CHIPR(DF,CHSQ)
   IMPLICIT REAL*8(A-H,O-Z)
   A=.5*DF
   X=.5*CHSQ
   IF(X)101,101,100
101 CHIPR=1.
   RETURN
100 TERM=1.
   SUM = 0.
   COFN=A
   IF(13.-X)110,110,120
110 IF(A-X)140,140,120
120 CON=1.
   FACT=-A
130 TEMP=SUM
   SUM=SUM+TERM
   COFN=COFN+1.
   TERM=TERM*X/COFN
   IF(SUM-TEMP)160,160,130
140 CON=0.
   FACT=X
150 TEMP=SUM
   SUM=SUM+TERM
   COFN=COFN-1.
   RATIO=COFN/X
   TERM=TERM*RATIO
   IF(SUM-TEMP)160,160,150
160 CHIPR=CON+DEXP(DLOG(SUM)-X+A*DLOG(X))/FACT
   RETURN
   END

```

APPENDIX E

COMPUTER PROGRAMS FOR CALCULATING THE LC50 AND 95% CONFIDENCE INTERVAL

3. TRIMMED SPEARMAN-KARBER PROGRAM

This program was written in FORTRAN PLUS IV v.3, at the Fisheries Bioassay Laboratory, Montana State University, Bozeman, Montana, by M.A. Hamilton and S.M. Hinkins, and is discussed in the publication: Hamilton, M.A., R.C. Russo, and R.V. Thurston, 1977, Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays, Environ. Sci. Technol. 11(7):714-719. The program is interactive and prompts the user for data input.

Note: Decimals must be used with data on concentration values and numbers of animals.

EXAMPLE

A. INPUT:

Enter the number of concentrations: 6
Do you want automatic trim calculations (yes or no): yes
Enter each concentration in ascending order: 3.125
6.25
12.5
25.0
50.0
100.0

Are the number of animals equal in all concentrations? Yes

Enter the number animals per concentration: 20.

Enter the number of animals that died in each concentration: 0.
0.
0.
8.
18.
20.

B. OUTPUT:

The printout includes the following information:

1. Raw data:

Concentrations: 3.125, 6.25, 12.5, 25.0, 50.0, 100.0

Number of organisms in each concentration: 20., 20., 20., 20., 20., 20.

Mortality in each concentrations: 0., 0., 0., 8., 18., 20.

2. Spearman-Karber Trim (Calculated) 0%

3. Spearman-Karber Estimates:

LC50 = 28.72

Lower 95% confidence limit = 24.03

Upper 95% confidence limit = 34.31

LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD

```

C* *****
C* THIS LISTING DESCRIBES THE FORTRAN SUBPROGRAMS FOR CALCULATING *
C* THE TRIMMED SPEARMAN-KARBER ESTIMATE OF THE LC50 WHICH IS IN *
C* USE AT THE FISHERIES BIOASSAY LABORATORY, MONTANA STATE UNIV., *
C* BOZEMAN AS OF JUNE, 1978. THE PROGRAMS WERE NOT ORIGINALLY *
C* WRITTEN FOR CIRCULATION AND HAVE NOT BEEN DOCUMENTED. THE *
C* USER WILL NEED TO WRITE A MAIN PROGRAM WITH APPROPRIATE INPUT- *
C* OUTPUT ROUTINES. THE FISHERIES BIOASSAY LABORATORY ASSUMES NO *
C* RESPONSIBILITY FOR THE TECHNICAL ACCURACY OF THESE PROGRAMS. *
C* PROGRAMMERS: M.A. HAMILTON AND S.M. HINKINS. *
C* *
C* MODIFIED ON JUNE 3, 1980 BY BRAD GREENWOOD FOR THE EPA AT DULUTH *
C* MINNESOTA TO ALLOW FOR AUTOMATIC TRIM OPTION. *
C* *
C* *
C* FOR REFERENCE, CITE: *
C* HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON. 1977. *
C* TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN *
C* LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS. ENVIRON. SCI. *
C* TECHNOL. 11(7): 714-719. CORRECTION 12(4): 417 (1978) *
C* -- *
C*****
C...CHKSK
      IMPLICIT REAL*8 (A-H,O-Z)
      INTEGER AMS
      DIMENSION X(10),KR(10),N(10),P(10),PT(10)
      DIMENSION XI(10) ! ERLD MODIFICATION
C--
C--
C--
      LOGICAL FLAGX,FLAGN,FLAGP,FLAGT,FLAGC,FLAGQ,FLAGLP
      LOGICAL*1 FLAG,YES,NO
      DATA YES,NO/'Y','N'/
C--
C--
C--
C MAIN PROGRAM TO PERFORM 100A TRIMMED SPEARMAN-KARBER CALCULATIONS
C CALLS MNTN AND SKA
C ANALYSIS OF LOG(CONCENTRATION)
C--
C--
C--
      FLAGX=.TRUE.
      FLAGN=.TRUE.
      FLAGP=.TRUE.
      FLAGQ=.TRUE.
      FLAGT=.TRUE.
      FLAGC=.TRUE.

```

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LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

```

FLAGLP=.FALSE.
C--
C--
C--
C***** 25.
C*      * 26.
C*  INSERT HERE THE CODE TO READ IN AND/OR  * 27.
C*  CALCULATE K, ( X(I),N(I), AND P(I) FOR  * 28.
C*  I=1,K), AND AA  * 29.
C*      * 30.
C--
C--
C--
      TYPE 10
      IF(FLAGLP) PRINT 10
10  FORMAT(' TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV',/)
      TYPE 20
20  FORMAT(' ENTER THE NUMBER OF DOSE CONCENTRATIONS: ',%)
      ACCEPT*,K
      IF( K .GT. 10 ) STOP ' ERROR. TOO MANY DOSES (MAX=10)---ABORTED'
11  CONTINUE
      IF(FLAGLP) PRINT 10
      IF( FLAGC ) TYPE 31
31  FORMAT(' DO YOU WANT AUTOMATIC TRIM CALCULATION (YES OR NO):',%)
      IF( FLAGC ) ACCEPT 210,FLAG
      IF((FLAG.EQ.YES).AND.FLAGC) FLAGT=.FALSE.
      IF((FLAG.EQ.YES).AND.FLAGC) AA=0.000
      IF( FLAGT ) TYPE 30
30  FORMAT(' ENTER THE % TRIM REQUESTED: ',%)
      IF( FLAGT ) ACCEPT*,AA
      IF(FLAGC.OR.FLAGT) AA=AA*.01 : CONVERT TO %
      IF( FLAGX ) TYPE 40,K
40  FORMAT(' ENTER THE',I3,' DOSE CONCENTRATIONS (IN INCREASING ORDER).',
1 /10X,'? ',%)
      IF( FLAGX ) ACCEPT*,(X1(I),I=1,K)
      IF(FLAGQ) FLAG=NO
      IF( FLAGQ ) TYPE 51
51  FORMAT(' ARE THE NUMBER OF ANIMALS IN EACH TANK EQUAL?',%)
      IF( FLAGQ ) ACCEPT 210,FLAG
      IF((FLAG.EQ.YES).AND.FLAGQ) FLAGN=.FALSE.
      IF((FLAG.EQ.YES).AND.FLAGQ) TYPE 52
52  FORMAT(' ENTER THE NUMBER FISH PER TANK:',%)
      IF(FLAGQ.AND.(FLAG.EQ.YES)) ACCEPT*,KT
      IF(FLAGQ) GO TO 53
60  TO 55
53  CONTINUE
      DO 54 I=1,K
          N(I)=KT

```


LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

```

54 CONTINUE
55 CONTINUE
IF( FLAGN ) TYPE 50
50 FORMAT(' ENTER THE TOTAL NUMBER OF ANIMALS IN EACH DOSE EXPOSURE.',
1 /10X,'? ', '$)
IF( FLAGN ) ACCEPT*,(N(I),I=1,K)
IF( FLAGP ) TYPE 60
60 FORMAT(' ENTER THE TOTAL NUMBER OF ANIMALS ',
1 ' THAT EXPIRED IN EACH DOSE.',/10X,'? ', '$)
IF( FLAGP ) ACCEPT*,(P(I),I=1,K)
DO 12 I=1,K
X(I)=X1(I)
12 CONTINUE : PRESERVE THE ORIGINAL DOSES.
C--
C--
C--
C*****
DO 1 I=1,K
PT(I)=P(I)/DFLOAT(N(I))
IF(X(I).NE.0.0) X(I)=DLOG(X(I))
1 CONTINUE
C.....ADJUST PROPORTIONS TO BE MONOTONE NONDECREASING.....
CALL MNTN(K,N,PT)
C...CALL 100A TRIMMED SPEARMAN-KARBER ESTIMATOR SUBPROGRAM
KERROR=0
2000 CONTINUE
IF(KERROR .EQ. 1) AA=AA+.01D0
IF(AA.GE.0.5D0) GO TO 555
KERROR=0
CALL SKA(ENU,VMU,KERROR,AA,K,X,N,PT)
IF(KERROR .EQ. 1) GO TO 2000
SD=DSQRT( DABS(VMU) ) : ERLD MODIFICATION
ELO=ENU-2.D0*SD
EUP=ENU+2.D0*SD
C*****
C*
C* IF DESIRED, INSERT HERE THE CODE TO OUTPUT THE
C* ESTIMATES,C.I.,ETC ON THE NATURAL LOG SCALE
C*
C*****
SK=DEXP(ENU)
ELO=DEXP(ELO)
EUP=DEXP(EUP)
C*****
C*
C* INSERT HERE THE CODE TO OUTPUT THE ESTIMATE OF
C* LC50(SK) AND THE ESTIMATED 95 CONFIDENCE INTERVAL
C* (ELO & EUP).

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LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

```

C*                                     *                               59.
C--
C--
C--
  TYPE 90
  IF(FLAGLP) PRINT 90
  90 FORMAT(// ' RAW DATA:')
  TYPE 91,(X1(I),I=1,K)
  IF(FLAGLP) PRINT 91,(X1(I),I=1,K)
  91 FORMAT(' DOSE:',T20,10F7.2)
  TYPE 92,(N(I),I=1,K)
  IF(FLAGLP) PRINT 92,(N(I),I=1,K)
  92 FORMAT(' NUMBER:',T20,10I7)
  TYPE 93,(P(I),I=1,K)
  IF(FLAGLP) PRINT 93,(P(I),I=1,K)
  93 FORMAT(' MORTALITIES:',T20,10F7.2)
  AAA=AA*100.
  TYPE 94,AAA
  IF(FLAGLP) PRINT 94,AAA
  94 FORMAT(' SPEARMAN-KARBER TRIM (CALCULATED): ',F7.2,'X')
  TYPE 100,SK,ELO,EUP
  IF(FLAGLP) PRINT 100,SK,ELO,EUP
  100 FORMAT(/1X,' SPEARMAN-KARBER ESTIMATES: LC50',T50,F12.2,
    1 / '          95% LOWER CONFIDENCE',T50,F12.2,
    2 / '          95% UPPER CONFIDENCE',T50,F12.2,
    3 /1X,80(' '),//)
C--
C--
C--
  IF(FLAGLP) GO TO 101
  99 CONTINUE
  TYPE 102
  102 FORMAT(' ', ' DO YOU WANT A COPY SENT TO THE PRINTER? ',0)
  ACCEPT 210,ANS
  IF(ANS.NE.'N'.AND.ANS.NE.'Y') GO TO 99
  IF(ANS.EQ.'N') GO TO 101
  FLAGX=.FALSE.
  FLAGC=.FALSE.
  FLAGN=.FALSE.
  FLAGP=.FALSE.
  FLAGQ=.FALSE.
  FLAGLP=.TRUE.
  FLAGT=.FALSE.
  GO TO 11
  101 CONTINUE
  TYPE 200
  200 FORMAT(' WOULD YOU LIKE TO MODIFY SOME OF YOUR DATA? ',0)
  ACCEPT 210,FLAG

```

LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

```

210 FORMAT(A1)
  IF( FLAG .EQ. NO ) STOP ' RUN COMPLETED '
C
C
  FLAGX=.FALSE.
  FLAGC=.FALSE.
  FLAGN=.FALSE.
  FLAGP=.FALSE.
  FLAGQ=.FALSE.
  FLAGLP=.FALSE.
  FLAGT=.FALSE.
  TYPE 220
220 FORMAT(' DO YOU WANT TO MODIFY ANY OF THE DOSES (YES,NO)? ',%)
  ACCEPT 210,FLAG
  IF( FLAG .EQ. YES ) FLAGX=.TRUE.
  TYPE 230
230 FORMAT(' DO YOU WANT TO MODIFY THE NUMBER OF ANIMALS? ',%)
  ACCEPT 210,FLAG
  IF( FLAG .EQ. YES ) FLAGN=.TRUE.
  TYPE 240
240 FORMAT(' DO YOU WANT TO MODIFY THE MORTALITIES? ',%)
  ACCEPT 210,FLAG
  IF( FLAG .EQ. YES ) FLAGP=.TRUE.
  TYPE 250
250 FORMAT(' DO YOU WANT TO CHANGE THE TRIM (YES,NO)? ',%)
  ACCEPT 210,FLAG
  IF( FLAG .EQ. YES ) FLAGT=.TRUE.
  GO TO 11
C--
C--
C*****
  555 WRITE ( 5,500) AA
  CALL EXIT
  500 FORMAT (F8.4,' SK NOT CALCULABLE')
  STOP
  END
C* MNTN
  SUBROUTINE MNTN(K,N, PT)
C* M. HAMILTON * DEPT. OF MATH. * MONTANA STATE UNIVERSITY*BOZEMAN *
C SUBROUTINE TO ADJUST PROPORTIONS TO BE MONOTONE INCREASING
C***K=NO. OF DOSE LEVELS
C***N(I)=NO. OF SUBJECTS AT DOSE I
C***PT(I)=PROPORTION OF RESPONSE AT DOSE I; ENTERS AS OBSERVED
C*** PROPORTION AND RETURNS AS ADJUSTED PROPORTION
C * * * * *
  IMPLICIT REAL*8 (A-H,O-Z)
  DIMENSION N(10),PT(10)
  7 CONTINUE

```

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78.

LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

NS=0	79.
I=0	80.
16 CONTINUE	81.
I=I+1	82.
DD=PT(I+1)-PT(I)	83.
IF(DD .GT. -1.0D-8) GO TO 8	84.
KA = I	85.
IF (I .EQ. 1) GO TO 10	86.
J=I+1	87.
20 CONTINUE	88.
J=J-1	89.
IF(DABS(PT(J)-PT(J-1))) .GT. 1.0D-8) GO TO 10	90.
KA=J-1	91.
IF (J .LE. 2) GO TO 21	92.
GO TO 20	93.
21 CONTINUE	94.
10 KB=I+1	95.
IF(I .EQ.(K-1)) GO TO 12	96.
I1=I+1	97.
K1=K-1	98.
DO 11 J=I1,K1	99.
DD=PT(J+1)-PT(J)	100.
IF(DD .LT. 1.0D-8) GO TO 11	101.
KB = J	102.
GO TO 12	103.
11 CONTINUE	104.
12 CONTINUE	105.
NCUM=0	106.
CUM=0.0D0	107.
DO 13 J=KA,KB	108.
CUM = CUM + PT(J)*N(J)	109.
13 NCUM = NCUM + N(J)	110.
DO 14 J=KA,KB	111.
14 PT(J)=CUM/NCUM	112.
I=KB	113.
NS=1	114.
8 IF (I .LE. (K-2)) GO TO 16	115.
IF (NS .GT. 0) GO TO 7	116.
RETURN	117.
END	118.
C** SKA	119.
SUBROUTINE SKA(SKAEST,VARSKA,KERROR,A,K,Z,N,PT)	120.
C** M. HAMILTON * DEPT. OF MATH. * MONTANA STATE UNIVERSITY * BOZEMAN *	121.
C* SUBROUTINE SKA CALCULATES THE 100*A PERCENT TRIMMED SPEARMAN-	122.
C***KARBER ESTIMATE OF THE MEDIAN OF THE TOLERANCE DISTRIBUTION.	123.
C***IT IS ASSUMED THAT THE TOLERANCE DISTRIBUTION IS SYMMETRIC.	124.
C***100*A PERCENT ARE TRIMMED FROM EACH TAIL.	125.
C***THE EST. VARIANCE OF THE ESTIMATE IS CALCULATED (VARSKA)	126.

LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

```

C***K=NO. OF DOSE LEVELS 127.
C***Z(I)=DOSE LEVEL I, ENTERED IN INCREASING ORDER 128.
C***N(I)=NUMBER OF SUBJECTS AT DOSE Z(I) 129.
C***PT(I)=PROPORTION OF RESPONSE AT DOSE Z(I); NONDECREASING IN I 130.
C***PT=P-TILDA OF E,S, & T (1977) PAPER 131.
C*****CALLS SUBROUTINE SK( )***** 132.
C***** 133.
      IMPLICIT REAL*8 (A-H,O-Z) 134.
      DIMENSION P(10),Z(10), N(10),PT(10) 135.
      NL=1 136.
      NU=K 137.
C...CHECK FOR CALCULABILITY 138.
      DIF=PT(NL)-A 139.
      IF (PT(NL) .LT. (1.D0-PT(NU))) DIF=1.D0-A-PT(NU) 140.
D TYPE *, ' GOT TO 140'
D TYPE *, ' 1.D0-A-PT(NU)=',1.D0-A-PT(NU)
D TYPE *, ' PT(NL)-A=',PT(NL)-A
D TYPE *, ' DIF=',DIF
      IF (DIF .GT. 0.0D0) GO TO 10 141.
C...FOR A=0.5, WHEN ONE OR MORE OF PT(I)=0.5, 142.
C.....SKAEST=MEAN OF ASSOCIATED DOSE LEVELS 143.
C.....VARSKA IS NOT CALCULATED AND IS SET TO ZERO 144.
      IF (DABS(A-0.5D0) .GT. 1.0D-8) GO TO 4 145.
      ZHU=0.0D0 146.
      NU=0 147.
      DO 5 I=1,K 148.
        IW=1 149.
        IF (DABS(PT(I)-A) .GT. 1.0D-8) IW=0 150.
        ZHU=ZHU+Z(I)*IW 151.
      5 NU=NU+IW 152.
      IF (NU .EQ. 0) GO TO 4 153.
      SKAEST=ZHU/NU 154.
      VARSKA=0.0D0 155.
D TYPE *, ' GOT TO 1ST RETURN LINE 155'
      RETURN 156.
C***** 157.
      4 CONTINUE 158.
      I=NU-1 159.
      9 CONTINUE 160.
      DD = A-PT(I) 161.
      IF( (DD.GT. -1.0D-8) .AND. (PT(I+1) .GT. A) ) GO TO 3 162.
      I=I-1 163.
D TYPE *, ' GOT TO 163'
      IF (I .LT. NL) GO TO 10 164.
      GO TO 9 165.
      3 JLT=I 166.
C...JLT=MAX(I SUCH THAT PT(I) .LE. A) 167.
C***** 168.

```


LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

```

      IF(DABS(A-0.5) .GT. 1.0D-8) GO TO 2          169.
      SKAEST=Z(JLT)+(Z(JLT+1)-Z(JLT))*(A-PT(JLT))/(PT(JLT+1)-PT(JLT)) 170.
      VARSKA=0.0                                  171.
C...WHEN A = 0.5, VARSKA IS NOT CALCULATED AND IS SET TO ZERO          172.
D TYPE*, ' GOT TO 2ND RETURN LINE 172'
      RETURN                                      173.
C*****                                           174.
      2 CONTINUE                                  175.
      NL1=NL+1                                    176.
      DO 6 I=NL1,NU                               177.
      KOUNT = I                                   178.
      DD = PT(I) - (1.D0-A)                      179.
      IF((PT(I-1) .LT. (1.D0-A) ) .AND. (DD .GT. -1.D-8)) GO TO 7    180.
      6 CONTINUE                                  181.
D TYPE*, ' GOT TO LINE 181'
      GO TO 10                                     182.
      7 JUT=KOUNT                                  183.
C...JUT=MIN(I SUCH THAT P(I) .GE. 1-A)          184.
C**CALL SUBROUTINE SK( )*****                   185.
D TYPE*, ' GOT TO 185,JLT=JUT'
      IF(JLT .EQ. JUT) GO TO 10                   186.
      CALL SK(SKAEST,VARSKA,Z,N,PT,A,JLT,JUT)    187.
D TYPE*, ' GOT TO 3RD RETURN AFTER CALL TO SK-LINE 187'
      RETURN                                      188.
      10 KERROR=1                                 189.
      RETURN                                      190.
      END                                         191.
C** SK                                           192.
      SUBROUTINE SK(SKEST,VARSK,Z,N,PT,A,KLT,KUT) 193.
C** M. HAMILTON * DEPT. OF MATH. * MONTANA STATE UNIVERSITY*BOZEMAN * 194.
C SUBROUTINE SK CALCULATES THE 100A TRIMMED SPEARMAN-KARBER          195.
C ESTIMATE AND THE ESTIMATED VARIANCE OF THE ESTIMATE                196.
C                                                                    197.
      IMPLICIT REAL*8 (A-H,O-Z)                  198.
      DIMENSION Z(10),N(10),PT(10)              199.
      IF (KLT .EQ. (KUT-1)) GO TO 2              200.
      IF (KLT .EQ. (KUT-2)) GO TO 3              201.
C...CALCULATIONS FOR (KUT-KLT) .GE. 3          202.
      Y1=(Z(KLT+1)-Z(KLT))*(A-PT(KLT))*(PT(KLT+1)-A) 203.
      Y1=Y1/(2.D0*(PT(KLT+1)-PT(KLT)))          204.
      Y2=(Z(KLT)+Z(KLT+1))*(PT(KLT+1)-A)/2.D0    205.
      Y4=(Z(KUT-1)+Z(KUT))*(1.D0-A-PT(KUT-1))/2.D0 206.
      Y5=(Z(KUT)-Z(KUT-1))*(1.D0-A-PT(KUT-1))*(PT(KUT)-1.D0+A) 207.
      Y5=Y5/(2.D0*(PT(KUT-1)-PT(KUT)))          208.
      Y3=0.D0                                    209.
      KLT1=KLT+1                                  210.
      KUT2=KUT-2                                  211.
      DO 4 I=KLT1,KUT2                            212.
      4 Y3=Y3+(Z(I)+Z(I+1))*(PT(I+1)-PT(I))/2.D0 213.
      SKEST=(Y1+Y2+Y3+Y4+Y5)/(1.D0-2.D0*A)      214.

```

LISTING OF COMPUTER PROGRAM FOR THE TRIMMED SPEARMAN-KARBER METHOD (CONT.)

```

V1=(Z(KLT+1)-Z(KLT))*(((PT(KLT+1)-A)/(PT(KLT+1)-PT(KLT))))**2)      215.
V1=(V1**2)*PT(KLT)*(1.D0-PT(KLT))/(N(KLT)*4.D0)                      216.
V2=(Z(KLT+1)-Z(KLT))*(((A-PT(KLT))/(PT(KLT+1)-PT(KLT))))**2)        217.
V2=Z(KLT)-Z(KLT+2)+V2                                                  218.
V2=(V2**2)*PT(KLT+1)*(1.D0-PT(KLT+1))/(N(KLT+1)*4.D0)              219.
V4=(Z(KUT)-Z(KUT-1))*(((PT(KUT)-1.D0+A)/(PT(KUT)-PT(KUT-1))))**2)    220.
V4=Z(KUT-2)-Z(KUT)+V4                                                  221.
V4=(V4**2)*PT(KUT-1)*(1.D0-PT(KUT-1))/(N(KUT-1)*4.D0)              222.
V5=(Z(KUT)-Z(KUT-1))*(((1.D0-A-PT(KUT-1))/(PT(KUT)-PT(KUT-1))))**2)  223.
V5=(V5**2)*PT(KUT)*(1.D0-PT(KUT))/(N(KUT)*4.D0)                    224.
V3=0.D0                                                                225.
IF (KUT .EQ. (KLT+3)) GO TO 8                                          226.
KLT2=KLT+2                                                             227.
KUT2=KUT-2                                                             228.
DO 9 I=KLT2,KUT2                                                       229.
9 V3=V3+((Z(I+1)-Z(I-1))**2)*PT(I)*(1.D0-PT(I))/(4.D0*N(I))        230.
8 VARSK=(V1+V2+V3+V4+V5)/((1.D0-2.D0*A)**2)                          231.
GO TO 10                                                                232.
C...CALCULATIONS FOR (KUT-KLT) .EQ. 1                                  233.
2 CONTINUE                                                             234.
SKEST=Z(KLT)+(0.5D0-PT(KLT))*(Z(KUT)-Z(KLT))/(PT(KUT)-PT(KLT))      235.
V1=((1.D0-2.D0*PT(KUT))**2)*PT(KLT)*(1.D0-PT(KLT))/N(KLT)          236.
V2=((1.D0-2.D0*PT(KLT))**2)*PT(KUT)*(1.D0-PT(KUT))/N(KUT)          237.
V3=((Z(KUT)-Z(KLT))/(2.D0*(PT(KUT)-PT(KLT))**2))**2                238.
VARSK=V3*(V1+V2)                                                       239.
C *** WARNING ***                                                     240.
C      IF PT(KLT)=0.0 &/OR PT(KUT)=1.0 THEN                          241.
C      VARSK IMMEDIATELY ABOVE IS INAPPROPRIATE                      242.
GO TO 10                                                                243.
C...CALCULATIONS FOR (KUT-KLT) .EQ. 2                                  244.
3 Y1=(A-PT(KLT))*(PT(KLT+1)-A)*(Z(KLT+1)-Z(KLT))                    245.
Y1=Y1/(2.D0*(PT(KLT+1)-PT(KLT)))                                      246.
Y2=(Z(KLT)+Z(KLT+1))*(PT(KLT+1)-A)/2.D0                             247.
Y3=(Z(KLT+1)+Z(KLT+2))*(1.D0-A-PT(KLT+1))/2.D0                     248.
Y4=(1.D0-A-PT(KLT+1))*(PT(KLT+2)-1.D0+A)*(Z(KLT+1)-Z(KLT+2))      249.
Y4=Y4/(2.D0*(PT(KLT+2)-PT(KLT+1)))                                   250.
SKEST=(Y1+Y2+Y3+Y4)/(1.D0-2.D0*A)                                    251.
V1=(Z(KLT+1)-Z(KLT))*(((PT(KLT+1)-A)/(PT(KLT+1)-PT(KLT))))**2)    252.
V1=(V1**2)*PT(KLT)*(1.D0-PT(KLT))/(4.D0*N(KLT))                    253.
V2=(Z(KLT)-Z(KUT))/2.D0                                               254.
VV=(Z(KUT)-Z(KLT+1))/2.D0                                             255.
V2=V2+VV*(((PT(KLT+2)-1.D0+A)/(PT(KLT+2)-PT(KLT+1))))**2)        256.
VV=(Z(KLT+1)-Z(KLT))/2.D0                                             257.
V2=V2-VV*(((A-PT(KLT))/(PT(KLT+1)-PT(KLT))))**2)                  258.
V2=(V2**2)*PT(KLT+1)*(1.D0-PT(KLT+1))/N(KLT+1)                     259.
V3=(Z(KUT)-Z(KLT+1))*(((1.D0-A-PT(KLT+1))/(PT(KUT)-PT(KLT+1))))**2  260.
V3=(V3**2)*PT(KUT)*(1.D0-PT(KUT))/(4.D0*N(KUT))                    261.
VARSK=(V1+V2+V3)/((1.D0-2.D0*A)**2)                                  262.
10 RETURN                                                             263.
END                                                                    264.

```

APPENDIX E

COMPUTER PROGRAM FOR CALCULATING THE LC50 AND 95% CONFIDENCE INTERVAL

4. PROGRAM FOR STATISTICAL COMPARISON OF LC50s

This program is written in BASIC PLUS 2, version 1.61, by James Dryer and determines whether two LC50 values are significantly different by comparing the two LC50 values and their upper confidence limits using the standard error of mean differences. The program is based on the report by Sprague and Fogels (1977), "Watch the Y in Bioassay," published in the Proceedings of the 3rd Aquatic Toxicity Workshop, Halifax, Nova Scotia, November 2-3, 1976, Environmental Protection Service Technical Report No. EPS-5-AR-77-1, Halifax, Nova Scotia, Canada. pp. 107-118.

The program uses the two LC50s and their upper confidence limits (UL), and is based on the following relationships:

$$G = \sqrt{\left[\log_{10} \frac{UL(1)}{LC50(1)} \right]^2 + \left[\log_{10} \frac{UL(2)}{LC50(2)} \right]^2}$$

Where:

$$H = 10^G$$

$$Z = \frac{\text{Larger LC50}}{\text{Smaller LC50}}$$

If Z is larger than H, the LC50s are considered to be significantly different.

If Z is smaller than H, the difference between the LC50 values is not considered significant.

EXAMPLE

A. INPUT

LC50 of first test? 20.62
Upper Confidence Limit of First Test? 29.84
LC50 of second test? 29.48
Upper Confidence Limit of First Test? 35.23

The following calculations are carried out by the program:

$$G = \sqrt{\left[\log_{10} \frac{29.84}{20.62} \right]^2 + \left[\log_{10} \frac{35.23}{29.48} \right]^2}$$

$$H = 10G = 10 \cdot 0.1619 = 1.51$$

$$Z = \frac{29.48}{20.62} = 1.43$$

Results:

Z is smaller than H. Therefore, the LC50s are not considered to be significantly different.

B. OUTPUT

The following output is produced by the program:

"THE LC50 VALUES ARE SUFFICIENTLY CLOSE AND MAY BE
FROM THE SAME POPULATION"

H = 1.51

Z = 1.43

LISTING OF COMPUTER PROGRAM FOR STATISTICAL COMPARISON OF LC50s

```
10 INPUT "LC50 OF FIRST TEST",A
20 INPUT "UPPER CONFIDENCE LIMIT OF FIRST TEST",C
30 INPUT "LC50 OF SECOND TEST",B
40 INPUT "UPPER CONFIDENCE LIMIT OF SECOND TEST",D
50 E=(LOG10(C/A))*2
60 F=(LOG10(D/B))*2
70 G=SQR(E+F)
80 H=10*(G)
90 IF A>B THEN 120
100 Z=B/A
110 GO TO 130
120 Z=A/B
130 IF H>Z THEN 160
140 PRINT "THE LC50 VALUES ARE SUFFICIENTLY REMOVED AND ARE PROBABLY"
141 PRINT "FROM DIFFERENT POPULATIONS."
142 PRINT "A = ",H
143 PRINT "B = ",Z
150 GO TO 170
160 PRINT "THE LC50 VALUES ARE SUFFICIENTLY CLOSE AND MAY BE FROM THE "
161 PRINT "SAME POPULATION"
162 PRINT "A = ",H
163 PRINT "B = ",Z
170 END
```


APPENDIX E

COMPUTER PROGRAMS FOR CALCULATING THE LC50 AND 95% CONFIDENCE INTERVAL

5. TOXDAT MULTI-METHOD PROGRAM (BINOMIAL, MOVING AVERAGE, AND PROBIT METHODS)

This program is written in FORTRAN PLUS IV v.3, by Charles Stephan, Environmental Research Laboratory, USEPA, Duluth, Minnesota, and calculates the LC50 and 95% confidence limits by each of the following three methods:

1. Binomial Distribution
2. Moving Average
3. Probit

The program was obtained from the Environmental Research Laboratory, U. S. Environmental Protection Agency, Duluth, Minnesota.

NOTE: a. Data are input from highest to lowest concentration.

b. Decimals must be used for concentration values and number of organisms that died, but not for "Number of Concentrations."

EXAMPLE #1

A. INPUT:

Enter the number of concentrations - it should be greater than 1 ? 6

Enter in descending order the concentrations used in the test:

? 100.
? 50.
? 25.
? 12.5
? 6.25
? 3.125

Enter the number of animals exposed at each concentration:

? 20.
? 20.
? 20.
? 20.
? 20.
? 20.

Enter the number of organisms that died at each concentration:

? 20.
? 16.
? 11.
? 7.
? 2.
? 0.

B. OUTPUT:

Where do you want your output to go? On your terminal (TT) or line printer (LP)?

Enter TT or LP? TT

A printout of the results is as follows:

```
-----  
CONC.      NUMBER      NUMBER      PERCENT      BINOMIAL  
           EXPOSED     DEAD        DEAD        PROB.(%)  
100        20          20          100         .0000953674  
50          20          16           80         .590897  
25          20          11           55         41.1901  
12.5        20           7           35         13.1588  
6.25        20           2           10         .0201225  
3.125       20           0            0         .0000953674
```

THE BINOMIAL TEST SHOWS THAT 6.25 AND 50 CAN BE
USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT
CONFIDENCE LIMITS SINCE THE ACTUAL CONFIDENCE LEVEL
ASSOCIATED WITH THESE LIMITS IS 99.389 PERCENT.
AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 21.0569

>>>>>>RESULTS CALCULATED USING THE MOVING AVERAGE METHOD

SPAN	G	LC50	95 PERCENT CONFIDENCE LIMITS	
5	.05135	19.865	15.6704	25.5024

>>>>>>RESULTS CALCULATED USING THE PROBIT METHOD

ITERATIONS	G	H	GOODNESS OF FIT PROBABILITY
6	.0838854	1	.730927

SLOPE = 2.67833

95 PERCENT CONFIDENCE LIMITS = 1.90261 AND 3.45405

LC50 = 20.2931

95 PERCENT CONFIDENCE LIMITS = 15.5785 AND 26.539

LC1 = 2.74538

95 PERCENT CONFIDENCE LIMITS = 1.16044 AND 4.52059

EXAMPLE #2

A. INPUT:

The following data are entered as described in Example #1:

Effluent Concentration	Number of Organisms Exposed	Number of Organisms That died
100.	20.	20.
50.	20.	18.
25.	20.	8.
12.5	20.	0.
6.25	20.	0.
3.125	20.	0.

B. OUTPUT:

The printout of the results is as follows:

```

-----
CONC.      NUMBER      NUMBER      PERCENT      BINOMIAL
          EXPOSED      DEAD        DEAD        PROB. (Z)
100        20         20         100         .0000953674
50          20         18          90         .0201225
25          20          8          40         25.1722
12.5        20          0           0         .0000953674
6.25        20          0           0         .0000953674
3.125       20          0           0         .0000953674
THE BINOMIAL TEST SHOWS THAT 12.5 AND 50 CAN BE
USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT
CONFIDENCE LIMITS SINCE THE ACTUAL CONFIDENCE LEVEL
ASSOCIATED WITH THESE LIMITS IS 99.9798 PERCENT.
AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 28.3375

>>>>>>RESULTS CALCULATED USING THE MOVING AVERAGE METHOD
SPAN      6          LC50      95 PERCENT CONFIDENCE LIMITS
4          .0313499      29.7135      24.135      37.2718
>>>>>>RESULTS CALCULATED USING THE PROBIT METHOD

ITERATIONS  0          H          GOODNESS OF FIT PROBABILITY
8          .156151      1          .958014

SLOPE =      5.96063
95 PERCENT CONFIDENCE LIMITS = 3.60523 AND 8.31602

LC50 =      28.9644
95 PERCENT CONFIDENCE LIMITS = 24.0946 AND 34.7947
LC1 =      11.7897
95 PERCENT CONFIDENCE LIMITS = 6.29974 AND 15.8096
-----

```

SUMMARY OF RESULTS:

A. Example #1

(1) Binomial Test

LC50 = 21.1
Upper 95% confidence limits = 50.0
Lower 95% confidence limits = 6.25

(2) Moving Average

LC50 = 19.9
Upper 95% confidence limits = 25.5
Lower 95% confidence limits = 15.7

(3) Probit

LC50 = 20.3
Upper 95% confidence limits = 24.4
Lower 95% confidence limits = 16.9

A. Example #2

(1) Binomial Test

LC50 = 28.3
Upper 95% confidence limits = 50.0
Lower 95% confidence limits = 12.5

(2) Moving Average

LC50 = 29.7
Upper 95% confidence limits = 37.3
Lower 95% confidence limits = 24.1

(3) Probit

LC50 = 29.0
Upper 95% confidence limits = 34.8
Lower 95% confidence limits = 24.1

LISTING OF TOXDAT MULTI-METHOD PROGRAM

```

10 DIM A(10),B(10),C(10),D(10),E(10),F(10),G(18,18),H(10),I(10),J(10),K(10)
20 DIM L(10),R(10),N(10),P(10),T(10),V(10),X(10),Y(10),Z(10)
30 INPUT "NUMBER OF CONCENTRATIONS =" ;M
40 FOR J=1 TO M
50 PRINT "CONCENTRATION NUMBER";J;" = "
51 INPUT C3
52 C(J)=C3
60 NEXT J
70 FOR J=1 TO M
80 PRINT "NUMBER OF FISH AT START OF TEST IN CONCENTRATION ";C(J);" = "
81 INPUT E(J)
90 NEXT J
100 FOR J=1 TO M
110 PRINT "NUMBER OF FISH DEAD IN CONCENTRATION ";C(J);" = "
111 INPUT D(J)
120 NEXT J
130 PRINT "CONC.", "NUMBER", "NUMBER", "PERCENT", "BINOMIAL"
140 PRINT " ", "EXPOSED", "DEAD", "DEAD", "PROB.(Z)"
150 K0=0
160 FOR J=1 TO M
170 L(J)=LOG10(C(J))
180 P(J)=D(J)/E(J)
190 I(J)=1
240 B1=0
250 N1=E(J)
260 IF P(J)>.5 THEN 290
270 N2=D(J)
280 GO TO 300
290 N2=E(J)-D(J)
300 FOR N=0 TO N2
310 B2,B3=1
320 FOR I=(N1-N+1) TO N1
330 B2=B2*I
340 NEXT I
350 FOR I = 2 TO N
360 B3=B3*I
370 NEXT I
380 B1=B1+(B2/B3)
390 NEXT N
400 B(J)=100*B1*(0.5^N1)
410 PRINT C(J),E(J),D(J),100*P(J),B(J)
450 A1=SQR(D(J)/(E(J)+1))
460 A2=ATN(A1/(SQR(1-A1*A1)))
470 IF D(J)=E(J) THEN 510
480 A3=SQR((D(J)+1)/(E(J)+1))
490 A4=ATN(A3/(SQR(1-A3*A3)))
500 GO TO 520
510 A4=1.57079633

```


LISTING OF TOXDAT MULTI-METHOD PROGRAM (CONT.)

```

520 A(J)=180*(A2+A4)/(2*PI)
530 NEXT J
540 FOR J=1 TO N
550 IF P(J)>0 THEN 610
560 IF J>1 THEN 590
570 Y(J)=-2.67*(1-P(J+1)/2)
580 GO TO 810
590 Y(J)=-2.67*(1-P(J-1)/2)
600 GO TO 810
610 IF P(J)<1 THEN 665
620 IF J<N THEN 650
630 Y(J)=2.67*(1-(1-P(J-1))/2)
640 GO TO 810
650 Y(J)=2.67*(1-(1-P(J+1))/2)
660 GO TO 810
665 P1=P(J)
670 I1=0
680 IF P1<=0.5 THEN 710
690 P1=1-P1
700 I1=1
710 P2=SQR(LOG(1/P1/P1))
720 P3=((0.010328*P2+0.802853)*P2+2.515517)
730 Y(J)=P3/(((0.001308*P2+0.189269)*P2+1.432788)*P2+1)-P2
740 IF I1=1 THEN 750
745 GO TO 760
750 Y(J)=-Y(J)
760 IF K0>0 THEN 790
770 X3=C(J)
780 GO TO 800
790 IF X3=C(J) THEN 810
800 K0=K0+1
810 NEXT J
1000 B4,B5,B6,B7=0
1010 FOR J=1 TO N
1020 IF B(J)<1776 THEN 1040
1030 GO TO 1230
1040 IF P(J)<.5 THEN 1090
1050 IF B(J)>2.5 THEN 1090
1060 B4=C(J)
1070 B5=B(J)
1080 NEXT J
1090 FOR J=N TO 1 STEP -1
1100 IF P(J)>.5 THEN 1150
1110 IF B(J)>2.5 THEN 1150
1120 B6=C(J)
1130 B7=B(J)
1140 NEXT J
1150 B8=100-B5-B7

```

LISTING OF TOXDAT MULTI-METHOD PROGRAM (CONT.)

```

1160 IF B4>0 THEN 1190
1170 PRINT "THE BINOMIAL TEST SHOWS THAT ";B6;"AND + INFINITY CAN BE"
1180 GO TO 1200
1190 PRINT "THE BINOMIAL TEST SHOWS THAT ";B6;"AND ";B4;" CAN BE"
1200 PRINT "USED AS STATISCALLY SOUND CONSERVATIVE 95 PERCENT"
1210 PRINT "CONFIDENCE LIMITS SINCE THE ACTUAL CONFIDENCE LEVEL"
1220 PRINT "ASSOCIATED WITH THESE LIMITS IS ";B8;" PERCENT."
1230 FOR J=1 TO M
1240 IF P(J)<0.5 THEN 1270
1250 G1=J
1260 NEXT J
1270 FOR J=M TO 1 STEP -1
1280 IF P(J)>0.5 THEN 1310
1290 G2=J
1300 NEXT J
1310 IF P(G2)<P(G1) THEN 1340
1320 M1=((C(G1))*(C(G2)))^-.5
1330 GO TO 1370
1340 G3=(45-A(G2))/(A(G1)-A(G2))
1350 G4=L(G2)+(L(G1)-L(G2))*G3
1360 M1=10^G4
1370 PRINT "AN APPROXIMATE LC50 FOR THIS SET OF DATA IS ";M1
1380 PRINT
1390 IF K0>1 THEN 1440
1400 PRINT "WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT"
1410 PRINT "DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE NOR THE"
1420 PRINT "PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS."
1430 GO TO 3850
1440 I4=0
1450 FOR S=M-1 TO 1 STEP -1
1460 FOR N=1 TO M-S+1
1470 IF S<3 THEN 1580
1480 FOR J=1 TO S-2
1490 J(J+2)=((L(N+J+1)-L(N+1))/(L(N+1)-L(N)))-J
1500 NEXT J
1510 FOR J=1 TO S
1520 T(J)=-.5*(S-1)+(J-1)+J(J)
1530 NEXT J
1540 W1,W2=0
1550 FOR K=1 TO S
1560 W1=W1+T(K)*(0.5+E(N+K-1))
1565 W2=W2+T(K)*T(K)*(0.5+E(N+K-1))
1570 NEXT K
1580 K1=1
1590 V(N)=0
1600 FOR J=N TO N+S-1
1610 IF S>2 THEN 1640
1620 M(J)=1

```

LISTING OF TOXDAT MULTI-METHOD PROGRAM (CONT.)

```

1630 GO TO 1650
1640 M(J)=W2-T(K1)*W1
1650 G(J,N)=M(J)*(0.5+E(J))
1660 V(N)=V(N)+G(J,N)
1670 K1=K1+1
1680 NEXT J
1690 K(N)=0
1700 F(N)=0
1710 FOR J=N TO N+S-1
1720 K(N)=K(N)+G(J,N)*L(J)/V(N)
1730 F(N)=F(N)+G(J,N)*A(J)/V(N)
1740 NEXT J
1750 F(N)=(INT((1E6)*F(N)+0.5))/(1E6)
1760 NEXT N
1770 K2=1
1780 FOR J=1 TO M-S
1790 X(K2)=0
1800 K4=0
1810 FOR N=J TO J+S
1820 IF P(N)=0 THEN 1850
1830 IF P(N)=1 THEN 1850
1840 K4=K4+1
1850 NEXT N
1860 IF K4<2 THEN 1920
1870 IF F(J)=F(J+1) THEN 1920
1880 IF F(J)<45 THEN 1920
1890 IF F(J+1)>45 THEN 1920
1900 X(K2)=J
1910 K2=K2+1
1920 NEXT J
1930 IF X(1)=0 THEN 2360
1940 IF I4=1 THEN 1980
1950 PRINT ">>>>>>>RESULTS CALCULATED USING THE MOVING AVERAGE METHOD"
1960 PRINT "SPAN","G","LC50","95 PERCENT CONFIDENCE LIMITS"
1970 I4=1
1980 FOR N=1 TO K2-1
1990 P=X(N)
2000 Q=X(N)+1
2010 Y=F(Q)-F(P)
2020 A=(45-F(P))/Y
2030 M2=K(P)+(K(Q)-K(P))*A
2040 M3=10^M2
2050 V1,V2,V3=0
2060 FOR J=1 TO S
2070 V1=V1+((3282.81*(G(P+J-1,P)^2))/((4*E(P+J-1)+2)*(V(P))^2))
2080 V2=V2+((3282.81*(G(Q+J-1,Q)^2))/((4*E(Q+J-1)+2)*(V(Q))^2))
2090 IF J=1 THEN 2110
2100 V3=V3+((3282.81*G(P+J-1,P)*G(Q+J-2,Q))/((4*E(P+J-1)+2)*V(P)*V(Q)))

```

LISTING OF TOXDAT MULTI-METHOD PROGRAM (CONT.)

```

2110 NEXT J
2120 V4=V1+V2-2*V3
2130 V5=V1-V3
2140 Z=1.96
2150 G=Z*Z*V4/(Y^2)
2160 IF G=1 THEN 2330
2170 R=V1-2*A*V5+(A^2)*V4-G*(V1-((V5^2)/V4))
2180 IF R<0 THEN 2330
2190 V6=Z*SQR(R)/(Y*(1-G))
2200 V7=(A-G*V5/V4)/(1-G)
2210 L2=K(P)+(K(Q)-K(P))*(V7-V6)
2220 U2=K(P)+(K(Q)-K(P))*(V7+V6)
2230 L3=10^L2
2240 U3=10^U2
2250 IF G<1 THEN 2310
2260 IF A<(V5/V4) THEN 2290
2270 PRINT S,G,M3,"0",U3
2280 GO TO 2340
2290 PRINT S,G,M3,L3,"+ INFINITY"
2300 GO TO 2340
2310 PRINT S,G,M3,L3,U3
2320 GO TO 2340
2330 PRINT S,G,M3," 0","+ INFINITY"
2340 NEXT M
2350 GO TO 2420
2360 NEXT S
2370 IF I4=1 THEN 2420
2380 PRINT "THE MOVING AVERAGE METHOD CANNOT BE USED WITH THIS SET OF DATA"
2390 PRINT "BECAUSE NO SPAN WHICH PRODUCES MOVING AVERAGE ANGLES THAT BRACKET"
2400 PRINT "45 DEGREES ALSO USES TWO PERCENT DEAD BETWEEN 0 AND 100 PERCENT."
2410 PRINT
2420 IF K0>1 THEN 2470
2430 PRINT "WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT"
2440 PRINT "DEAD IS BETWEEN 0 AND 100, THE PROBIT METHOD CANNOT GIVE ANY"
2450 PRINT "STATISTICALLY SOUND RESULTS."
2460 GO TO 3850
2470 S1,S2,S3,S4,K3,I3=0
2480 FOR J=1 TO M
2485 S1=S1+L(J)
2490 S2=S2+Y(J)
2500 S3=S3+L(J)*L(J)
2510 S4=S4+L(J)*Y(J)
2520 NEXT J
2530 B=(S4-S1*S2/M)/(S3-S1*S1/M)
2540 A=(S2-B*S1)/M
2550 F1,F2,F3,F4,F5,F6=0
2560 FOR J=1 TO M
2570 Z(J)=A+B*L(J)

```

LISTING OF TOXDAT MULTI-METHOD PROGRAM (CONT.)

```

2580 Z1=ABS(Z(J))
2590 IF Z1>8 THEN 2700
2600 Z2=0.39894228*EXP(-.5*Z1*Z1)
2610 Z3=1/(1+0.2316419*Z1)
2620 Z4=((1.330274*Z3-1.821256)*Z3+1.781478)*Z3
2630 P=Z2*Z3*((Z4-.356563782)*Z3+.31938153)
2640 IF Z(J)<0 THEN 2680
2650 Q=P
2660 P=1-P
2670 GO TO 2730
2680 Q=1-P
2690 GO TO 2730
2700 P=1E-16
2710 Z2=1E-15
2720 GO TO 2640
2730 W3=E(J)*(Z2/P)*(Z2/Q)
2740 W4=Z(J)+(P(J)-P)/Z2
2750 F1=F1+L(J)*W3
2760 F2=F2+W3*W4
2770 F3=F3+L(J)*L(J)*W3
2780 F4=F4+W3*W4*W4
2790 F5=F5+L(J)*W3*W4
2800 F6=F6+W3
2810 NEXT J
2820 F7=F5-F1*F2/F6
2830 F8=F3-F1^2/F6
2840 B=F7/F8
2850 A=(F2-B*F1)/F6
2860 K3=K3+1
2870 D1=0
2880 FOR J=1 TO N
2890 D1=(A+B*L(J)-Z(J))^2+D1
2900 NEXT J
2910 IF D1<1E-11 THEN 2960
2920 IF K3<25 THEN 2550
2930 PRINT "NO CONVERGENCE IN 25 ITERATIONS. THE PROBIT METHOD PROBABLY"
2940 PRINT "CANNOT BE USED WITH THIS SET OF DATA."
2950 GO TO 3850
2960 F9=F1/F6
2970 F0=F4-F2^2/F6
2980 C1=F0-F7^2/F8
2990 V=M-2
3000 IF V=0 THEN 3430
3010 IF C1/V>20 THEN 3220
3020 J=1
3030 FOR I=V TO 2 STEP -2
3040 J=J+1
3050 NEXT I

```


LISTING OF TOXDAT MULTI-METHOD PROGRAM (CONT.)

```

3060 C2=C1*(INT((V+1)/2))*EXP(-C1/2)/J
3070 IF INT (V/2)=V/2 THEN 3100
3080 C3=SQR(2/C1/PI)
3090 GO TO 3110
3100 C3=1
3110 C4,C5=1
3120 V=V+2
3130 C5=C5*C1/V
3140 IF C5<1E-10 THEN 3170
3150 C4=C4+C5
3160 GO TO 3120
3170 C6=1-C2*C3*C4
3180 IF C6>=0.001 THEN 3200
3190 C6=0
3200 V=M-2
3210 GO TO 3250
3220 C6=0
3230 GO TO 3250
3240 C6=1492
3250 IF C6<=0.05 THEN 3290
3260 T5=1.96
3270 H=1
3280 GO TO 3370
3290 IF V=1 THEN 3330
3300 IF V=2 THEN 3350
3310 T5=1.95996+1/(.413*V-.423)
3320 GO TO 3360
3330 T5=12.706
3340 GO TO 3360
3350 T5=4.303
3360 H=C1/V
3370 G=H*T5^2/B^2/F8
3380 E1=SQR(H/F8)
3390 E2=B-T5*E1
3400 E3=B+T5*E1
3410 PRINT ">>>>>>RESULTS CALCULATED USING THE PROBIT METHOD"
3415 PRINT
3420 PRINT"ITERATIONS","G","H","GOODNESS OF FIT PROBABILITY"
3430 IF C6=1492 THEN 3460
3440 PRINT K3,G,H,C6
3450 GO TO 3470
3460 PRINT K3,G,H," (CANNOT BE CALCULATED)"
3470 PRINT
3480 IF C6>0 THEN 3510
3490 PRINT "A PROBABILITY OF 0 MEANS THAT IT IS LESS THAN 0.001"
3500 PRINT
3510 IF C6>0.05 THEN 3550
3520 PRINT "SINCE THE PROBABILITY IS LESS THAN 0.05, RESULTS CALCULATED"

```

LISTING OF TOXDAT MULTI-METHOD PROGRAM (CONT.)

```

3530 PRINT "USING THE PROBIT METHOD PROBABLY SHOULD NOT BE USED."
3540 PRINT
3550 PRINT "SLOPE = ",B
3560 PRINT "95 PERCENT CONFIDENCE LIMITS = ";E2; " AND ";E3
3570 PRINT
3580 M4=-A/B
3590 M5=10^(M4)
3600 PRINT "LC50 = ",M5
3610 IF G=1 THEN 3770
3620 H1=H*((1-G)/F6+(M4-F9)^2/F8)
3630 IF H1<0 THEN 3770
3640 H2=SQR(H1)
3650 L4=M4+G*(M4-F9)/((1-G)-H2*T5/((ABS(B))*(1-G)))
3660 U4=M4+G*(M4-F9)/((1-G)+H2*T5/((ABS(B))*(1-G)))
3670 L5=10^L4
3680 U5=10^U4
3690 IF G<1 THEN 3750
3700 IF M4>F9 THEN 3730
3710 PRINT "95 PERCENT CONFIDENCE LIMITS = 0 AND " ;U5
3720 GO TO 3790
3730 PRINT "95 PERCENT CONFIDENCE LIMITS = ";L5; " AND + INFINITY"
3740 GO TO 3790
3750 PRINT "95 PERCENT CONFIDENCE LIMITS = " ;L5; " AND " ;U5
3760 GO TO 3790
3770 PRINT "95 PERCENT CONFIDENCE LIMITS = 0 AND + INFINITY"
3790 IF I3=1 THEN 3850
3800 M4=(-2.32679-A)/B
3810 M5=10^M4
3820 PRINT "LC1 = ",M5
3830 I3=1
3840 GO TO 3610
3850 GO TO 10
3860 END

```